

In what state is the Union?

President George Bush's State of the Union address last week included enough of the right things to pass muster, but it remains to be seen whether his recipes will work, now and in the more distant future.

If it is the job of a president of the United States to sense his or her people's mood and, if necessary, to lighten it, President George Bush did well in his State of the Union message last week. To the surprise and even the consternation of the world outside, the United States is now plunged into such a slough of introspection and uncertainty that it tends to be forgotten that, less than a year ago, the United States was celebrating its leadership in a brilliant military victory in the Middle East. More than that, as Bush kept saying on Tuesday last week, "the Cold War has been won". So why has the recession not melted away? Why, from Detroit to Silicon Valley, do once-daring innovators sense what they think of as "Japan" breathing down their necks? Why, after more than three years of unprecedented popularity in the opinion polls, is Bush himself anxious about what may happen between now and election day in November? What has gone wrong?

Questions such as these are imponderable. Would the recession have seemed to matter less if the Gulf War had dragged on? Would Japanese competitiveness seem less daunting if there were still a clamant need to build more nuclear warheads and, more expensively, their delivery systems? But, put that way, the questions can be answered only in the negative. A protracted Gulf War would eventually have seemed like Vietnam, while strategic weapons that (by definition) are never used lack the popular appeal of the latest snazzy products from Sony and Toyota. Lucky Bush, that the two wars have ended. His job would have been more difficult if they had dragged on. And, even now, there is only promise to go on.

Peace dividend

What peace dividend does the ending of the Cold War bring? Bush's plan to freeze some strategic weapons at their present strength and to cancel others will subtract \$50,000 million from the defence budget in the next five years. That is a lot, but not much of a dent in the annual federal deficit, now estimated at \$400,000 million. And, like Russia's President Boris Yeltsin (who announced his own unilateral strategic cuts the day after Bush), the Administration has not yet swung the axe seriously at the huge cost of the military establishment. He will not be surprised if the Congress tries to do that for him.

Part of the difficulty is that servicemen demobilized are almost indistinguishable (except by the medical benefits they enjoy) from those laid off from factories: they enlist

in the army of the unemployed, at least temporarily. And that is where the rub comes. Indeed, even the cancellation of strategic weapons will have some of that effect. What will they be thinking in Palmdale, California, the home of the B-2 bomber, for example? Beating swords into ploughshares may be an uplifting occupation, but also unrewarding if there is no demand for ploughshares. Bush's consolation is that Yeltsin's identical problem is at once bigger (by a factor of at least three) and has been made more difficult to solve by the rigid habits of three-quarters of a century.

Recession

So, inevitably, the US Administration is to wage war on recession. That was the slogan of last week's address. Indeed, there are to be two wars — one short-term, the "kick-start" of the past several weeks, and one long-term. Last week's short-term recipe is in many ways ingenious, not least by arranging that the Internal Revenue Service will more accurately subtract taxpayers' dues from their salaries, so putting more money into circulation. Otherwise, there are tax-breaks for families (an extra \$500 deducted from annual salaries before calculating tax) and for first-time house-buyers. It will be interesting to see whether the recipe works. It deserves a chance, but nobody should complain if it fails. In its present mood, the United States is more concerned to pay its debts than to rush off to the shops. Keynesian orthodoxy suggests that, in its present pickle, the Administration would borrow money to restart the US economy. The trouble is that it cannot; the endemic federal deficit is too big for that.

Is the outcome of the long-term war any more certain? It depends. Bush asks that the tax incentives for spending on research and development should become permanent, which is a good idea. His cabinet colleagues have been whispering about a National Technology Initiative, but nobody yet quite knows what it is. Rightly and constructively, Bush hopes for long-term benefits from the North American Free Trade Agreement (Canada and Mexico with the United States); now that the United States is inextricably locked into world trade, exports that can compete internationally are sinews of survival. The slogan of competitiveness has been about since President Jimmy Carter's time; the question that neither the Administration nor US business has yet faced is whether it makes sense to hope to breathe new life into yesterday's suc-



cesses — the industries represented by Bush's companions in Japan the other week.

Bush is also right to declare, as he did last week, that the United States needs better public education and general health insurance of some kind if it is to make the best use of its people, and to give them what they want. Nobody will quarrel about the medicine prescribed, but there will be endless argument about the means of its delivery. In any case, as the rest of the world knows, these are areas in which publicly directed social engineering is as tricky and difficult as attempts by civil servants to second-guess the ideal development of industry.

So must uncertainty persist? Not necessarily. For one thing, Bush reaffirmed last week the Administration's commitment to research. That is splendid, and as it should be; let us hope that the Congress gives Bush what he asks for (see page 486). By good luck, Bush also has the world's most flexible economy to sustain him, a splendid record of even recent innovation in technical fields that matter (or will matter) a great deal and an ebullient people. The short-term battle against recession may take longer than anybody is yet telling, but the long-term war is winnable, provided that a few conditions can be met. First, the federal deficit must be made to melt away, even if that means cutting back on the Strategic Defense Initiative, the space station Freedom and even the Superconducting Super Collider. Second, public education deserves even more attention than Bush promised in his address three years ago, when he seemed bent on becoming the "education president". Why has so little happened since the need was first identified and clearly articulated a decade ago?

Finally, the United States needs to play a trick on itself, with the help of Bush or his successor. With France, it enjoys a constitution in which personal liberty is eloquently enshrined. But the lower parts of the social heap have recently become uncomfortably conspicuous for everybody. Social problems, from drugs to homicide, undermine not merely peace of mind but the capacity of the US to function as the unitary state it used to be. Before President Lyndon Johnson was submerged by Vietnam, he lifted people's spirits with the slogan of "the Great Society". Nobody would expect Republican Bush to sing the same tune, but the problems are the same and there needs to be a tune. Will Bush or anybody find one before November? Presumably that is what the election is about. □

Election fever

There seem to be elections everywhere, but the British version promises little for science.

EVERYWHERE, there seems an election around the corner. France, for example, will hold municipal elections in May, followed by parliamentary elections next year (if not sooner). The United States, whose predictable electoral cycle is thereby also protracted, is already in the thick of a campaign concluding only in November. Britain, where the timing of elections is in the gift of the prime minister

and his party, must hold an election before mid-July, but will probably be at the polls in early April. The incumbent governments in the United States and France will be banging the drum for research: France's encouragement of research in the past decade may be the Mitterrand governments' only lasting achievement, while President George Bush last week pinned his faith in a restoration of US competitiveness on federal research spending (and tax incentives). By contrast, but as expected, little has been heard as yet in Britain.

That is a shame, reflecting the universal embarrassment of British politicians about an issue they do not understand. Gone are the days when the then Mr Harold (later Lord) Wilson was elected prime minister in 1964 on a promise of prosperity by "white-hot technology": his Labour successors offer instead a sensible pattern of what they call training — and have been galled to find that the government party has stolen their prospectus (for which there is little yet to show). The politicians' difficulty is not with the Wilson doctrine, but their puzzlement that it seems not to apply in Britain. The explanation is not that researchers are layabouts and indifferent to national needs, even if that hypothesis of Mrs Margaret Thatcher's has shaped British policy on research for a decade. The fault lies instead with British industry and the economic climate in which it seeks survival. Why else has not British industry been more daring on the strength of know-how garnered from elsewhere, as Japanese companies are often wrongly said to have prospered (see page 493)? It is not that British research's needs of its paymasters are obscure. This journal's *Manifesto for British Science* (*Nature* **353**, 105; 1991) has spelled out many of them.

The academic sector exists to recruit able people and to educate them in research, but its practitioners are grossly underpaid, as are the students they recruit. That state of affairs compromises Britain's future, not only because educated and skilled people go elsewhere, but because recruitment lags. The link between the academic sector and industrial innovation is something else again. With the exception of those in the pharmaceutical industry, British companies have slipped into skimping on long-term research and development, often quoting investors' short-term demands as their excuse. So governments and their opponents are hunting for means of presenting industry with innovative technology, neatly bundled in tasteful gift-wrapping. That is one reading of President Bush's National Technology Initiative, given an airing last week. The British equivalent may well be the content of a paper prepared for the Engineering Council by Dr John Fairclough, previously chief scientific adviser at the Cabinet Office, who advocates sector-based research institutes along the lines of Germany's Fraunhofer institutes. Before the government seizes on this to fill out its thin election manifesto, it should reflect that its predecessors stopped supporting industrial research associations as recently as the 1970s and that Japanese success in recent decades hangs on the close integration of research and development within the structure of a company. □

Genome project faces commercialization test

- US investor recruiting leading researchers
- Company aims to dominate gene sequencing

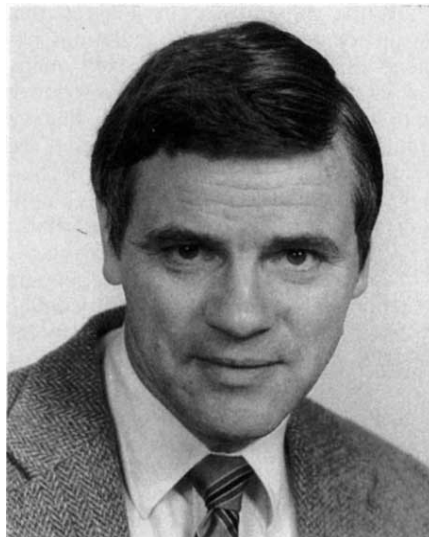
Washington & London

ARMED with tens of millions of dollars in capital, a private investor is making the first serious bid to commercialize the Human Genome Project. Frederick Bourke, a wealthy entrepreneur, is on a worldwide search for top genetic researchers to join a new company he plans to set up in Seattle, Washington. With state-of-the-art technology and the best talent money can buy, he aims to dominate the effort to sequence the estimated 3,000 million chemical bases of the human genome, as well as those of other species. The heads of the two teams now leading the effort to sequence the nematode, *Caenorhabditis elegans* are now in negotiations with Bourke and seriously considering leaving their academic posts to join the company, which has not yet been incorporated or named.

Robert Waterston, at the Washington University in St Louis and John Sulston of the UK Medical Research Council (MRC) Laboratory of Molecular Biology at Cambridge — the two principal collaborators in the \$6 million worm genome project — say they are convinced that it is time to move to the next step in the effort, the development of advanced sequencing technology and eventual production-scale automation. As a highly repetitive task,

large-scale gene sequencing is tailor-made for a company. Furthermore, moving this sort of 'production line' work out of academic laboratories frees university resources for basic research, they say.

The fact that Bourke might make a



Hood's technology may launch a \$50 million company.

hefty profit by essentially monopolizing the high-efficiency gene sequencing mar-

ket may bother some genome purists, but many others see it as a natural evolution of the project. "It was inevitable that the genome project would be commercialized soon," says Maynard Olson, another Washington University geneticist who is on the US Genome Project advisory panel. "It's clear that the [sequencing side of the] project has to scale up in some way, and it's clear that it can't be done in an academic environment. I see no moral principal that one should turn one's back to efficient sequencing in industry."

Indeed, Olson is so taken by the concept that he has decided to leave St Louis to accept a position this summer in the new department of molecular biotechnology at the University of Washington, Seattle. Although plans have not been finalized, it appears certain that the new biotechnology department — created last year with a \$12 million gift from Microsoft co-founder William Gates — will be closely affiliated with Bourke's new company. Olson will take with him several members of his laboratory, and says that he expects to eventually have some scientific collaboration with the company.

The magnet behind this migration of leading scientists to Seattle is Leroy Hood, a gene sequencing pioneer who started Applied Biosystems Inc. (ABI), one of the largest makers of automated gene sequencing equipment. Last October, the University of Washington announced that, armed with the Gates endowment, it had recruited Hood and several members of his laboratory from the California Institute of Technology, where he is a professor and director of the university's National Science Foundation (NSF) Science

A turn of the worm

WHAT galls researchers most about the threat of a corporate grab of the project to map and sequence the genome of the nematode *C. elegans* is not just the possibility of a derailed effort to understand a model species, but the implication for the entire Human Genome Project. *C. elegans* research is one of the only truly international collaborations in the 15-year, \$3,000 million undertaking, and as such, has been something of a model itself.

Robert Waterston, at Washington University in St. Louis, and John Sulston at the UK Medical Research Council (MRC) Molecular Biology laboratory in Cambridge, are by all accounts one of the success stories of the genome project. They have been collaborating on the worm for five years, and in 1990 the US National Institutes of Health (NIH) and the MRC began an unprecedented three-year project to support jointly full-scale sequencing at the two laboratories. Total support is about \$6 million, of which

NIH provides two-thirds and MRC the rest. The money is spent equally at the two facilities.

The researchers are now about halfway through a pilot project to sequence three million of the 100 million bases in the worm genome. Given the success of the venture, and the conspicuous lack of similar collaboration elsewhere in the initiative, genome leaders would be sorry to see the worm researchers go.

"I like the idea of a big project that is shared by two countries," says James Watson, director of the NIH genome centre. "I hope that isn't lost." On the other side of the Atlantic, Dai Rees, MRC secretary, is facing the prospect of giving up a key part of one of relatively few research laboratories in which Britain is indisputably world-class. "We've put 20 years of investment into this project, and it's just reaching its culmination. It's just rather a pity if that's creamed off," he says. Rees is hoping to find enough additional money for the project to persuade Sulston to stay.

Waterston and Sulston both say that they have every intention of continuing their worm work, even if it is in a privately-held sequencing company in Seattle. "*C. elegans* would represent a beginning", both in learning how to sequence several hundred thousand bases a day and in interpreting those sequences, Waterston says. "That will help down the line, when you want to work on human DNA." The company will have to prove it can actually do real world sequencing, and *C. elegans* is as good a demonstration project as any, he points out.

But whether Waterston and Sulston will actually be able to finish the *C. elegans* genome in a corporate culture remains to be seen. There is, after all, little market for the nematode gene sequences themselves. Frederick Bourke, the entrepreneur who is recruiting the researchers, is hoping to turn a profit in five years. It is hard to imagine a worm standing in the way.

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and Technology Center for Molecular Biotechnology. Hood will chair the new University of Washington department, the university announced.

Although Hood's financial relationship with Bourke's company has not yet been decided, Bourke says he considers Hood a "co-founder". ABI will be a principal supplier of equipment for the operation. NSF has not decided if it will renew funding for his centre, which now gets about \$3.5 million a year from the agency. (Nine of the 11 Science and Technology centres were renewed last year, but a decision on the Caltech facility was delayed, pending resolution of Hood's status.) Mary Clutter, director of the NSF biology division, says that she expects Hood to submit an application for renewal this month. A Caltech official says that the university will not challenge Hood's intention to take the centre with him.

Some researchers are concerned that Hood's involvement with both the NSF centre and the company (which will presumably be competing for government contracts) could represent a conflict of interest. Bourke says he is aware of the concern and intends to avoid such a conflict. Hood declined to be interviewed.

Initially, Bourke is aiming to take a large slice of a contract sequencing market expected to be worth over \$100 million by the middle of the decade. But the eventual worth of a mapped and sequenced human genome is orders of magnitude beyond that. Isadore Edelman, who directs the human genome centre at Columbia University and is a principal collaborator with Bourke on the new company, says that the company's intention is ultimately to capitalize on the genetic information it obtains through sequencing, by producing gene-based diagnostic tools and therapeutics.

"I think that the ability to sequence DNA in the genome will become the next industrial revolution," says Bourke. "Being able to do something as basic as sequencing gives us a generic position in this revolution." He notes that Japan is well on its way to developing a gene sequencing effort as a collaboration between industry, government and academic institutions. That effort, now funded at about \$14 million, is also aimed at developing automated sequencing technology (see *Nature* 351, 593; 1991). "I'm concerned that we're being left behind," Bourke says.

Bourke expects start-up funding for his company to be around \$50 million. The company will eventually consist of at least three divisions: a large-scale sequencing effort operating on contract for the government and the pharmaceutical industry; a group developing database and computer technology to deal with the vast amounts of information the sequencing operation will generate; and a division to carry out company-initiative

genetic research. The last group, working with genetic mapping laboratories in academic institutions, will focus on finding genes and other genetic information that could be used in commercial products, such as diagnostics tests for genetic conditions and infectious disease such as AIDS.

Edelman hopes that within three to five years the company will be able to sequence as many as 200 million bases a year. Initial plans are for about 100 automated gene sequencing machines "about twice as good as the best now available," he says. Each would be able to sequence about 500,000 base a year, at a contract price of \$.25 to \$.50 each.

While most researchers agree that sequencing in academic institutions has generally not been working well, many are uneasy about Bourke's plans to move it to industry. Mostly, they worry that the company will sequence large parts of the human genome with the sole aim of finding genes and patenting them, thus claiming property rights to substantial portions.

Edelman says this will not be the case. "We're not trying to sequence the genome and dominate all of biology and medicine with a patent position." The company intends to publish its work, and only patent genes if their function has been determined and such patents are considered "acceptable practice", he says. Both Waterston and Sulston say they were adamant that the gene sequences the company finds remain in the public domain. "Rather to our surprise he [Bourke] was still interested," Sulston says.

Nevertheless, many researchers are taking a wait-and-see position. And it seems clear that, whatever the company does to change the commercial prospects of the genome, its impact on the genome project will be significant. If it can indeed sequence far more efficiently than an academic operation, the US genome project will be hard pressed to find an argument against giving it most of the sequencing work and retaining only genetic mapping and basic research for the universities. This is a blow for the project, which now must face the probability that the lion's share of its funding will probably soon go not to academic researchers, but to industrial operations like Bourke's.

UK officials take a more parochial view. Dai Rees, secretary of the UK Medical Research Council, says he would "be happy to contract out work to a British company," but not to Bourke's operation. "If the [worm] project goes to that company, then they can fund it," he says. Speaking in an unofficial capacity, Sir Walter Bodmer, president of the Human Genome Organisation, said he was personally concerned that if sequencing contracts start going to companies rather than academic institutions, then so will the spin-offs of technological development, which would presumably then be proprietary company

information.

For the worm genome community, which is now the most focused on sequencing of all the model-species genome projects, "this is going to be a major change in the power structure," says Christopher Fields, a NIH researcher. "I can imagine the *C. elegans* community being very threatened by this." Sulston says that even if he joins the company, he intends to maintain collaborations with his Cambridge colleagues on the worm genome, and the MRC is hoping to find extra money to encourage project researchers to stay in Britain (see page 483).

While the debate continues, Bourke is still recruiting. Among the researchers he has had meetings with are NIH researcher Craig Venter, David Lipman, who is a database expert at the NIH Library of Medicine, and C. Thomas Caskey, a Baylor College of Medicine geneticist, as well as members of Caskey's laboratory. "There's hardly a lab that he hasn't approached," says one researcher. So far, no one has signed on the dotted line. But if Waterston and Sulston do indeed make the move, others are expected to follow.

Christopher Anderson & Peter Aldhous

INDIRECT COSTS

Dingell probe expands

Washington

CONGRESS' continuing investigation into the overhead charges for federally-funded research has expanded to include contractors for Environmental Protection Agency (EPA) and the Department of Energy (DOE) weapons laboratories. Representative John Dingell (Democrat, Michigan) announced at a hearing last week that his investigations and oversight subcommittee was auditing some of the contractors and that "the initial results are starting to make the universities look like small potatoes." He has scheduled hearings on the new probe in March.

Officials from the two agencies — the Office of Naval Research and the Department of Health and Human Service — that monitor government research grants testified that they have now expanded their university investigations from a handful of high-profile institutions such as Stanford University and the Massachusetts Institute of Technology to virtually all of the 300 universities under their jurisdiction. They explained that even special agreements between certain universities and the government, that have been in effect for nearly a decade, are now under question. By law, the agreements (known as memoranda of understanding) must be "equitable". Many were not, said J. Dexter Peach of the congressional General Accounting Office, and cancelling them retroactively would not be a "changing of the rules", it would be a "shift in enforcement". **Christopher Anderson**

Will US rival strip BTG bare?

London

THE British government's plan to sell British Technology Group (BTG) has returned as a focus of political controversy. The new development is that BTG's principal international competitor, Research Corporation Technologies (RCT) of Tucson, Arizona, is bidding for a stake in BTG. Its involvement has sparked fears — hotly denied by RCT — that it may eventually launch a hostile takeover, with the intention of dismantling BTG and selling its patents.

Both groups specialize in patenting inventions, investing in their development and subsequently selling patent licences to manufacturing and other companies. Although many universities and government laboratories make their own arrangements, both BTG and RCT enjoy a wide-ranging portfolio of patents, often assigned by academic inventors, so they can group patents together to produce more lucrative licensing packages.

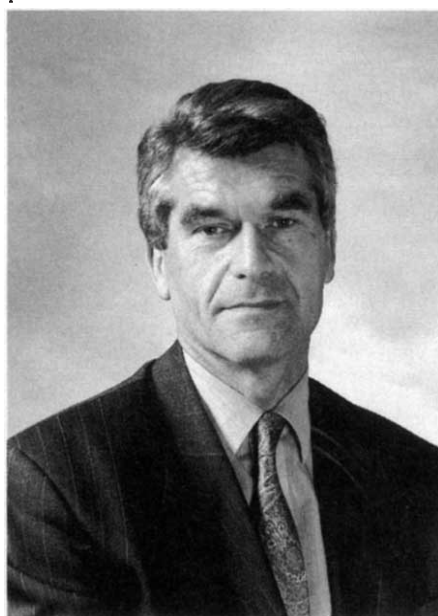
Because this market is dominated by the two companies, RCT's interest in BTG raises important questions of competition. And with BTG planning to expand into RCT's home market, having opened a US subsidiary (see *Nature* **352**, 652; 1991), the British Labour party believes that RCT's intention is to emasculate its competitor's US operations.

The Conservative government has said that BTG will be sold to a consortium of investors, with individual members — except under undefined "exceptional circumstances" — holding no more than a 15 per cent stake. The formula is supposed to stop BTG falling into the hands of investors who might be tempted to sell off some of its portfolio of patents for short-term profit. The accountants Price Waterhouse, who are handling the sale, have cast a veil of secrecy over the entire process, but it is thought that three consortia now stand as front-runners to buy BTG.

One consortium is headed by BTG's management and staff, headed by chief executive Ian Harvey (see *Nature* **355**, 102; 9 January 1992). A second is led by Sir Ronald Mason, formerly chief scientist at the Ministry of Defence, and comprises a number of industry-linked research associations. But it is a third group, including RCT and led by John Ashworth, director of the London School of Economics, that is now at the centre of a bitter political row. A report in the 26 January *Sunday Telegraph*, which said that RCT was seeking a 30 per cent shareholding, has horrified BTG's senior managers, and led the opposition Labour Party to demand a government statement on the sale. A Department of Trade and Industry spokesman says the 15 per cent limit will be applied flexibly, but adds that a

30 per cent holding is unlikely to be acceptable.

Labour's science and technology spokesman, Jeremy Bray, believes that RCT wants to curtail BTG's US operations. He points to an earlier approach RCT made to BTG's management, suggesting a joint management buyout. Under this plan — given short shrift by Harvey — BTG would have concentrated on the European market, allowing RCT to dominate in the United States. Bray also argues that the government's 'golden share' in BTG, a majority share that the government says it will retain for five years, to prevent a hostile takeover, offers little



Ashworth sees "xenophobia" in opposition to US buyer for BTG.

protection. A similar golden share, in the Jaguar motor company, was disposed of by the government as the Ford company was allowed to take control of the group.

The concern about RCT extends beyond the Labour party. Peter Doyle, research director of ICI, says that as "a concerned individual", he has to question RCT's intentions. "Do you think this adds to the scope for the licensing of new technologies [to industry], or is it more liable to lead to an elimination of competition?" he asks.

Doyle argues that BTG's present style of operation should be retained and is worried that this might not be the case with RCT as the dominant 'expert' shareholder. There are subtle differences between the two groups' styles. RCT, unlike BTG, is a not-for-profit corporation (which, incidentally, makes it immune to takeover). RCT also runs a much smaller staff than BTG, choosing not to retain in-house patent experts and lawyers, instead employing outside contractors to work on spe-

cific projects. RCT's business is also more finely focused on the licensing of technologies from academic institutions. Unlike BTG, it has no strong interest in 'corporate licensing' — selling access to technologies developed by one company to other industrial concerns.

Ashworth says that he would "like to bring over to Britain RCT's style and mode of operation", but denies that RCT's motivation is to protect itself from competition. RCT's president, Gary Munsinger, says that, in any case, BTG-USA is principally concerned with corporate licensing, so the two groups are not serious competitors in the US market. But BTG's medium-term plans include a drive to increase its uptake of technology from US universities, and Munsinger admits that he would like both groups to expand "based on inventions each originates from its own territory". Ashworth can give no firm assurances that BTG-USA would not be closed under his consortium's ownership, before he has studied its finances.

Ashworth's involvement with RCT has enraged the Labour party. During the passage of the BTG privatization bill through parliament, Labour cooperated with the Committee of Vice-Chancellors and Principals (CVCP) to secure an amendment to ensure that the universities, represented by CVCP, would be consulted about the sale. Labour politicians are incensed that a senior member of the CVCP should now emerge at the head of a bid they see as damaging to BTG.

Ashworth says that he withdrew from all CVCP discussions about BTG's privatization after he was approached by RCT — and accuses the British opponents of his consortium of barely concealed "xenophobia". Ashworth suggests that they compare RCT's record with that of BTG: RCT returns over 60 per cent of its revenues to its inventors, he says. BTG officials agree that BTG returns a smaller proportion of its licence income to inventors (around 25 per cent), but say that the group invests a far higher proportion of its revenues directly into new technologies — when this is taken into account, BTG's payments into academia compare well with those of RCT.

Even if the RCT-led bid is not successful, the Labour party claims that RCT will have gained commercially-sensitive information about BTG. Last Friday [31 January], Labour industry spokesman Gordon Brown demanded a government statement on "new evidence that BTG is being forced to hand over details of many of [its] technologies". Price Waterhouse have opened a 'data room' where the rival consortia can study documents about BTG to help them prepare their bids, but will not reveal the precise information being made available.

Peter Aldhous

Seven per cent solution: too good to be true?

Washington

LAST week President George Bush released his budget request for fiscal year 1993 (starting on 1 October 1992), in which he proposes that US basic research should get a seven per cent increase, to \$13,400 million. But as usual, there is no guarantee that the agencies will get anything like those generous figures.

Such is the paradox of the US budget process. Every year the president asks for a large increase for science, which is a popular measure to promote in a State of the Union address. Last year, for example, Bush asked for \$13,300 million, just slightly less than the figure he offered this year. But Congress, which has the real world to worry about, must actually find money to support the whole enterprise. By the time it had finished paying for the deficit and the bankrupt savings and loan institutions, it could only find \$12,500 million—\$800 million less than the request — to spend on basic research in 1992. In other words, the budget is a good place to watch for Administration priorities, but one should not count on the numbers.

With that *caveat*, these are some of the highlights of the proposed 1993 science budget:

Overall

With the general reduction in defence spending, it is tempting to look to the new budget for a corresponding shift of balance from defence research and development to the civilian side. Keen eyesight helps. The traditional 60:40 ratio of spending on defence and civilian research and development changes by exactly one percentage point in the President's proposal, to 59:41 — still "clearly insufficient" to spur enough research to help the economy, the House Science, Space and Technology Committee complains.

Lots of lobbying for funding to universities for 'small science' has apparently paid off, with a government-wide increase of nine per cent for individual investigators, mostly at the National Institutes of Health (NIH), the National Science Foundation (NSF) and the research programmes of the Department of Energy (DOE).

Increased emphasis on the commercial application of research is also a theme in this budget. Technology programmes under the Department of Commerce, including the traditionally underfunded National

Institute of Standards and Technology (26 per cent increase) and National Technical Information Service (12 per cent), are finally rising to the level Congress has sought. The Administration is also starting to catch Congress's enthusiasm for technology transfer, by requesting \$579 million for transfer programmes, including a 19 per cent increase for in-house technology transfer offices in federal agencies. And although it is only \$1 million, the Administration has at last provided some money for a Critical Technologies Institute, an office to identify and encourage emerging technologies that Congress has been pushing for (and is sure to endow further in the final budget).

Adding to the growing list of multi-

sciences directorate gets one of the largest increases within the agency, 25.5 per cent, to \$107 million. Individual investigators at NSF will receive 17 per cent more under the Administration plan.

National Institutes of Health

NIH's budget is set to rise 5.5 per cent, to \$8,900 million, with funding for individual investigators doing somewhat better at an increase of 7 per cent. AIDS research funding appears to have reached a plateau, with only a 4 per cent increase, although support for treatment would go up by 20 per cent.

The Human Genome Project, which NIH shares with DOE, is slated for a seven per cent rise, to \$175 million between the

two agencies. Although this is close to the \$200 million a year level that project officials had hoped to reach by now, the slight funding lag reflects the project's general difficulties in meeting the projections of its five-year plan.

Department of Energy

After the despair of last year's darkest moments of project-slashing, even a flat budget for DOE physics and energy research would have been received gratefully. In fact, the Administration did even better. High energy and nuclear physics are up 12 per cent, to \$1.650 million, which will allow the Relativistic Heavy Ion Collider and the Main Injector Ring at Fermilab to continue as planned.

Most of that increase, however, is due to the Superconducting Super Collider (SSC). Facing a congressional season in which the SSC will doubtless be attacked once more as the chief cause of the impoverishment of small science, Energy Secretary James Watkins went to some pains to point out that, since 1989, SSC spending has formed a rising wedge on top of a general research budget that has remained more or less constant. This year's SSC request is \$650 million.

But Watkins had a different explanation for the pressures on small science. If it were not for the appropriation of money by Congress to various pork-barrel projects, the department would not be forced to spend money that would otherwise go to good research on "bricks and mortar". He claimed that the research budget would be 15 per cent higher than it actually is, had Congress not raided it for pet programmes. An innovation in this year's budget, Watkins said, was the provision of a line item of \$50 million dedi-

US science budget highlights

figures in millions	1992 enacted	1993 proposal	Per cent increase
Basic Research			
Doubling the NSF budget by 1994	\$2,572	\$3,026	18
Support for Individual Investigators (NIH, NSF, DOE)	7,273	7,939	9
Human Genome Project	164	175	7
Superconducting Super Collider	484	650	34
Global Climate Change	1,110	1,372	24
Astronomy and Astrophysics	836	890	6
Competitive agricultural research	98	150	53
Applied Research			
High Performance Computing & Communications	655	803	23
Advanced Materials and Processing	1,659	1,821	10
Biotechnology	3,759	4,030	7
Energy R&D	774	914	18
Fusion	337	360	7
Advanced Manufacturing	252	321	27
Public Health	4,757	4,849	2
National Institute of Standards & Technology	247	311	26
Space Technology	273	305	12

agency science initiatives, the Administration is launching new programmes in biotechnology and advanced materials. The proposed increases for existing initiatives of climate change research and high-performance computing are 24 and 23 per cent respectively. White House science adviser D. Allan Bromley said that the Administration is considering adding advanced manufacturing to the research initiatives list next year.

National Science Foundation

NSF gets the largest increase of any science agency: 17 per cent (to \$3,030 million), which would hypothetically leave it on track for a promised budget in 1994 that is twice what it was getting in 1987. But \$60 million of the \$454 million increase is just to fix an accounting anomaly that put some of the Antarctic research programme under the Department of Defence. Recalculating, NSF would only get a 13.2 per cent rise. NSF's new social

cated to small science, which may not be cut without his approval.

National Aeronautics and Space Administration

The theme of this year's space budget is 'steady as she goes'. Total NASA spending planned for fiscal year 1993 stands at \$14,993 million, an increase of just 4.5 per cent over last year. Room has been made to increase support for Space Station Freedom, from \$2,030 million to \$2,250 million, and for the Earth Observing System, which will rise by over one-third to \$308 million. To pay for these outlays, a few wholesale cuts were made, notably the cancellation of the \$470 million Advanced Solid Rocket Motor programme, a deliberate challenge to its principal congressional supporter, Jamie Whitten (Democrat, Mississippi), chairman of the House appropriations committee. The Comet Rendezvous and Asteroid Fly-by (CRAF) mission and a shuttleborne relativity experiment are also eliminated.

The most difficult decision, said NASA administrator Admiral Richard Truly, was to cut CRAF. Its twin, Cassini (which is to visit Saturn), is retained, but with funding for Magellan's Venus-mapping mission scheduled to end, and with Galileo still Jupiter-bound but almost incommunicado because of the failure of its high-speed data transmitter, the US solar system exploration programme seems to be headed for a lean period. Only the US-European solar probe Ulysses is in space, on target, and working perfectly.

Biotechnology

In the face of stiff competition from other countries, principally Japan, whose government is targeting the biotechnology industry in much the same way it targeted the semiconductor industry, the Administration plans to give US biotechnology its own multi-agency initiative. By cobbling together all the existing federal research that has anything to do with biotechnology, and finding a small amount of new money, the budget proposes a \$4,030 million programme spread out over 12 agencies. This is, the Administration says, an increase of 7 per cent from 1992 funding levels. Although industrial investment in biotechnology shows no sign of letting up, the Administration believes that there is untapped potential in areas relating to manufacturing, bioprocessing, energy and the environment.

William Small, executive director of the Association of Biotechnology Companies, says the Bush initiative is a "positive first step, but only that", and said that fewer regulations and faster patent and product approvals are needed to bring products to the marketplace.

Christopher Anderson, David Lindley & Diane Gershon

At frontier's end, the "P" word

Washington

VANNEVAR Bush, the postwar US science advisor, called science "the endless frontier", and recommended, in short, that scientists be given money freely in the expectation that from this disinterested largesse technological and social benefits would necessarily flow. But in recent years, increasing austerity has made the future seem less than infinite. As Congress prepares to set the research budget for another year of grim economics, funding in many areas is expected to be flat or worse, and politicians are looking for projects to cut in order that the rest might be saved. But deciding which ones to axe and which to spare is turning out to be the usual muddle, due in part to the science community's traditional unwillingness to choose favourites.

For a ranking based on quality of science, there seems to be no one to ask but the scientists themselves. But they, for the most part, are not saying. The "P" word, priorities, is anathema; for most researchers, a priority ranking of research projects is perceived, usually rightly, as an execution list in reverse order. In a report* released last month by the National Academy of Sciences (NAS) on long-term space research planning, the NAS space studies board listed a half-dozen scary arguments why scientist avoid priorities like the plague. But for each argument it provided a counter-argument. The negative reason for scientists to set priorities is that if they do not, someone else will; the positive reason is that if scientists can indeed choose and then (the hard part) keep their consensus together, they may find themselves with real political power.

Of course, advice is easy to give, and the NAS report does no priority-setting of its own. The first phase of the study, now completed, aimed simply to make the case that setting priorities is a good idea. The second phase, now begun, is to "attempt to develop a credible methodology that the...community could use to recommend priorities." Clearly this priority-setting business is no picnic.

A revealing example of the perils of setting priorities appeared last month in a *Washington Post* series on Vice President Dan Quayle, head of the National Space Council, which was resurrected in 1989 to coordinate the Administration's space policy. The panel, which was composed of prominent scientists and aerospace executives, was preparing the "Augustine report" to recommend NASA's agenda for the next decade (see *Nature* 348, 569; 1990). According to the account of a key meeting, panel members were clear on where NASA's priorities should lie: basic science first, then technology, then environmental studies, then a new launch ve-

hicle, and finally the space station and a mission to Mars.

Fortunately for the two lowest-ranked programmes, Richard Darman, director of the White House Office of Management and Budget, was in attendance at the meeting. Nothing if not familiar with the ways of Washington, Darman warned the panel that listing something last was an invitation to kill it. In fact, he said, ranking things at all was only going to give Congress ammunition to undermine Bush's space policy.

The final report listed science as most importance, but lumped together all the other space efforts into a single "balanced" programme, without ranked priorities.

Even when scientists do fight the gag reflex and actually set priorities, they may not get what they want. Surveys by the astronomy community, conducted every ten years since 1970, have provided a solid political base for many of the country's large astronomical facilities. But the latest NAS astronomy survey, chaired by John Bahcall and released in March last year, listed as its top priority the \$1,300 million Space Infrared Telescope Facility (SIRTF), only to find that the NASA budget released last week mentions SIRTF nowhere. The project is simply too expensive to contemplate for now.

Asked last week what conclusion researchers should draw from seeing the astronomers' first pick cut altogether, White House science advisor Allen Bromley said the lesson is that scientists are "only one voice among many." They can either set priorities and be an articulate voice, he cautioned, or argue for more of everything and get lost in the noise.

At a meeting with the press last week, Representative George Brown (Democrat, California), chairman of the House Science, Space and Technology Committee, warned that the penalty to be paid for trying to stay above the political fray by not choosing priorities is that political earmarking will reign supreme. "I cannot overemphasize the importance of making scientific merit the first and chief criteria used to judge a research area...free of any other distillation or filtering," he said. "Without this primary ranking, policy makers lose their only opportunity for an unadulterated, peer-reviewed judgment of the science." The administration estimates that earmarking robs research of 15 per cent of its funding. Whether clearer priorities from the scientific community would have dissuaded Congress from indulging in its pork habit is not clear. But as Brown pointed out, it cannot hurt.

Christopher Anderson & David Lindley

* Setting Priorities for Space Research: Opportunities and Imperatives. Space Studies Board, National Research Council. 1991

Peers slam peer review

London

SYSTEMATIC biology, the classification of living things based on evolutionary relationships, is a field in decline due to the failure of the peer-review process in Britain to recognize its "immeasurable" importance, according to a report last week from the Science and Technology Committee of the House of Lords.

Research proposals in systematics submitted to the public grant-making research councils also seem to fall between cracks. The few applications that are still made to the Science and Engineering Research Council (SERC) tended to lose to "more innovative research approaches", the report finds.

It also complains that the Advisory Board for the Research Councils (ABRC), which oversees the research councils, has not chosen to intervene to direct funds to neglected fields. Ironically, the plight of British systematics was noted in the ABRC's own report *Taxonomy in Britain*, published in 1979.

But times have changed, says Neil Chalmers, director of the Natural History Museum (NHM), noting the prominence of green politics and the forthcoming UNCED conference in June; administrators may now be more inclined to follow recommendations on systematics, he believes. Michael Claridge, president of the Systematics Association and one of the committee's two academic advisers, agrees. Because the government will be obliged to reply to the report in writing, he believes politicians will be forced to pay attention to systematics.

On money, the committee recommends that core funding should be maintained in real terms and that ABRC should set aside an extra £1 million a year for five years, partly to support courses designed to attract new blood. The committee argues that systematics should then "take its place with other branches of science". Lord Dainton, chairman of the committee, says the five-year programme would "make or break" the field.

Total British spending on systematics has remained at about £30 million a year since 1980, and so has been a declining proportion of the total science budget, which has increased by 28 per cent in real terms since then. The committee also notes that 'core' funding for systematics research fell from £11.5 million in 1980 to £10.4 million in 1990, and that research council support fell by more than a third during the same period, from £3.3 million to £2.0 million.

Research manpower in museums also fell, between 1980 and 1990, by 25 per cent — even before the substantial job cuts at the NHM announced in 1990 which provoked the House of Lords inquiry.

There has also been a shift towards short-term contract employment; 99 per cent of museum systematists were permanently employed in 1980, compared with 66 per cent in 1990.

The proportion of time spent studying systematics by biology undergraduates has more than halved since 1980, from 17.1 to 8.9 per cent, in line with and perhaps because of the increased popularity of modular courses at British universities.

The changing age structure of university systematists is also, as Chalmers puts it, "very worrying": in 1980, 24 per cent of systematics teachers in universities were under 35 and 44 per cent were 46 or more. Just ten years later, only 8 per cent were under 35 and 63 per cent had passed 46. Plainly, experts are retiring or dying off and are not being replaced.

Museums employ the lion's share of systematists and thus consume most core funding. The NHM is now supported through the Office of Arts and Libraries (OAL), which has been much criticized for not appreciating the scientific work at NHM. Chalmers says he will continue to press for "level funding". The Lords committee also recommends that OAL should be advised by a science panel and that there should be a rolling fund of £0.5 million a year to support the curation of natural history collections at institutions such as local museums.

Curation is a strong theme in the House of Lords report. Many witnesses emphasized that the best-kept collections are those in which researchers take an active interest. This sentiment was prompted by moves at NHM to separate curation and research as distinct jobs. The report side-steps this issue, but recommends that no collection should be without the attention of an active researcher, even if part-time or peripatetic.

The committee also urges that bodies concerned with systematics should set up a forum to keep the situation under review, and to rationalize the present dispersed and disorganized roster of British collections. What the committee has in mind is an extension of the 1961 agreement dividing research and curation of specimens of the world's flora between NHM and the Royal Botanic Gardens at Kew. John Marsden, executive secretary of the Linnean Society of London, agrees with Claridge that "it's the only way to solve the enormous problem of documenting biological diversity".

Dainton (a chemist) says he is now firmly convinced of the potential benefits of systematic biology, attainable with modest increases in expenditure. "The cost of getting it right is small" he says: "the cost of getting it wrong is enormous."

Henry Gee

Jones's head rolls

London

DAVID JONES, the much-criticized general director of London Zoo, has emerged as the major casualty of the zoo's financial crisis. The Council of the Zoological Society of London, which runs the zoo, has decided to abolish Jones's post from 30 April. The zoo's two sites, the troubled Regent's Park site in central London, and Whipsnade Zoo, north of the capital, will in future be run by separate directors. A new management committee of society council members is expected to take a stronger role in defining the zoo's strategy.

Zoological Society fellows who last month inflicted a damaging vote of no confidence in their council's past performance, but stopped short of demanding the council's resignation after receiving assurances that the zoo's management would be overhauled (see *Nature* 355, 100; 9 January 1992), now claim an important victory, and believe the threat of closure for the Regent's Park site is receding.

Colin Tudge, a zoologist and writer and one of the leaders of the so-called 'Reform Group' of society fellows that proposed the no confidence motion, believes that Jones's departure is necessary to restore the zoo management's credibility. Jones, he says, was too closely associated with a series of expensive investment plans, mooted since the mid-1980s, that failed to secure the zoo's long-term financial future and eventually led to the threat of closure for the Regent's Park site.

Jones was also damaged by the unfavourable public reaction to the disclosure of the zoo's financial plight. At the time, it was suggested that zoo animals might have to be destroyed, if the UK government did not provide money to save the zoo. When it emerged that this slaughter was unlikely to happen, many supporters of the zoo expressed distaste at the zoo management's tactics.

Nevertheless, the society's council is considering setting up a new position of overseas director to allow Jones to remain affiliated with the zoo. Tudge is pleased that the council seems to have largely abandoned the expensive plans to relaunch the Regent's Park site around a series of 'themed' exhibits, which have been priced at between £12 million and £60 million. He says that the council's latest plans have borrowed heavily from the low-budget proposals put forward by the Reform Group, which place emphasis on the zoo's captive breeding programmes. Tudge believes the council's latest estimate to redevelop the site, of £9 million, may be reduced still further, increasing the likelihood that sponsors will come forward. A possible closure of the Regent's Park Zoo in the autumn "no longer seems to be on the agenda," he says. Peter Aldhous

Fighting the rules of the game

Nairobi, Kenya

ANIMAL geneticists in Kenya, in collaboration with colleagues in the United States, Denmark and other African countries, have embarked on an ambitious programme to attempt to preserve the genetic diversity of Kenya's wild animals. The wildlife genetics programme, organized by the National Museums of Kenya, with the Kenya Wildlife Service, has begun in response to changing wildlife management policies that regard wild animals as an economic resource and to changing social attitudes that regard zebras, gazelles, elephants and others as pests.

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Pests? Wild animals risk becoming an expendable commodity under Kenya's new rules.

Rashid Aman and Pieter Kat, wildlife geneticists at the Kenya Museums, hope to collect tissue samples from animals as they move or are moved around the country in order to keep track of species integrity and species mix. The programme was initiated as the two collaborated with Oliver Ryder of the Center for Reproduction of Endangered Species at the San Diego Zoo in California. The Kenyan Museums' Institute of Primate Research bought modern equipment with a grant from the European Communities. Ryder then amplified those funds with grant money from the Pew Charitable Trusts and the John and Beverly Stauffer Foundation to help establish in Kenya the laboratory techniques for growing cell lines for chromosomal analysis, DNA amplification and PCR analysis. The institute's first tissue sample came from the ear of a wounded elephant that had been treated by Wildlife Service veterinary surgeons.

Since then, Aman and Kat have added tissue samples from other elephants, wild dogs, cheetahs and bovid species. Meanwhile, Ryder is teaching techniques for genetic analysis to a Kenyan researcher

who is visiting his San Diego laboratory. At the Institute of Population Biology in Copenhagen, Denmark, two Kenyan technicians have recently completed a three-month training course in PCR and sequencing techniques. The Danish institute is collaborating with Aman and Kat on a study of interspecies genetic variation in bovids and intraspecies variation of bovids and elephants.

The need for wildlife genetics has become acute because Kenya is making plans to encourage game ranching, as well as big-game hunting. With 70 per cent of the country's wild animals living on private land—and an increasing attitude that they have to pay for their existence or be destroyed—the government recently approved a policy to allow people with permits to crop (raise, kill and the sell meat of) wild animals that are not endangered, such as zebras and gazelles. Twenty-five businesses have applied for permits, apparently hoping to copy the success of Kenya's current game ranch (the Hopcraft Ranch) which has made a name for itself selling the meat of giraffes, zebras, Thomson's and Grant's gazelles to the Carnivore, a well-known Nairobi restaurant that also serves vegetarian fare.

The new policy allows people to kill animals on their property for private use; it also permits the sale and transport of wild animals from one ranch to another.

Some people in Kenya are lobbying the government for the return of big-game hunting, while ranchers, whose crops are destroyed by elephants and whose cattle are attacked by hyenas and lions, want the government to corral such wild beasts into fenced national parks.

Either way, the natural traffic of wild animals will be affected. Hence the desire to gather crucial genetic information, about susceptibility or resistance to disease for instance, for the long-term success of animal conservation.

Despite good intentions, Aman has met resistance from animal welfare advocates who complain that the loud dart guns used to stun animals before obtaining tissue samples frighten elephants and other animals. Customs agents, who hold radioactive isotopes in custody so long that they are no longer scientifically useful, are another difficulty.

Nevertheless, the wildlife geneticists intend to prevail. They are now hoping to expand into informal collaborations with the many scientists who visit Kenya to study its wildlife. The Kenya Wildlife Service is beginning to ask such visiting researchers, as a condition for permission to work in Kenya, to collect samples for the wildlife genetics programme.

Jane Stevens

Tokyo quake

Tokyo

TOKYO'S 12 million residents were abruptly woken in the early hours of Sunday morning (2 February) by one of the biggest earthquakes to hit the city in several years. No serious damage occurred, but the earthquake has drawn attention to an unpredictable type of earthquake that can strike directly beneath the city at any time.

The quake, which measured 5.7 on the Richter scale, hit at 4:04 am with an epicentre about 90 km below Tokyo Bay next to the city. The earthquake occurred in a zone where three tectonic plates converge beneath the city. The Philippine Sea plate plunges below the Eurasian plate, on which Tokyo rests, at a depth of about 30 km. And the Philippine Sea plate is in turn underridden by the subducting Pacific plate at even greater depth.

Tokyo and surrounding areas have an extensive network of seismometers and other equipment run by various government agencies to try and predict major earthquakes. But the network is designed primarily to predict the so-called Tokai earthquake, which is expected to strike at any time along the edge of the Philippine plate in Suruga Bay about 120 km southwest of Tokyo. The network may also pick up precursors of a great earthquake in adjacent Sagami Bay, the site of the Great Kanto Earthquake of 1923 which destroyed much of Tokyo and Yokohama.

But even the most optimistic seismologists in Japan accept that prediction of earthquakes in the zone of three converging plates beneath Tokyo is beyond the present capabilities of science. These "directly underneath" or chokka-gata earthquakes, although generally not as large as the big Suruga or Sagami Bay earthquakes (which measure 7.5 to 8.0 on the Richter scale), can be devastating if they occur at shallow depth. Fortunately, Sunday's earthquake occurred deep down at 90 km.

David Swinbanks

CHINESE SCIENCE

Academy grows younger

Beijing

THE Chinese Academy of Sciences, the country's leading academic body, this month elected 210 new members, bringing its total complement to 515 and reducing the average age of its members from 75 to 69. The youngest of the new members is Zhao Yu-Fen, a 42-year-old woman chemist from Qinghua University. The election is the first since 1980, and marked the end of a 13-month selection process. Candidates were suggested by China's universities and research centres, and voted for by the academy's existing members. Like the Academy of Sciences in the former USSR, the Chinese Academy runs a large number of its own research institutes. You Qin Li

Wittgenstein and Smythies

SIR — Contrary to some comments^{1,2}, my recent letter in *Nature* was not an attack on Wittgenstein: nor did I ever claim he was insane³ (a purely legal term). As a (very) minor poet myself⁴, I do not feel it any insult to be called a (good) poet rather than a (poor) philosopher. Nor ought there to be any shame in being paranoid or schizophrenic. It may even be helpful, as in the case of Rousseau, who, "as every one knows, suffered a terrifying paranoid breakdown in 1767–8"⁵. This may have made him sensitive to certain negative aspects of human relationships, important for sociology, that most people prefer to ignore.

The Times in a leading article hauled me over the coals for so demeaning a great man as to call him paranoid. It seems to me that this shows a lamentable intolerance toward psychiatrically ill people: their achievements in spite of their illness should be admired.

My criticism was of those philosophers who misunderstand Wittgenstein. On the continent of Europe, 'philosophy' tends to involve an emotional and poetic attempt to grasp the 'essence of Being' or some such — a far cry from the traditions of Locke, Berkeley and Hume and their followers. The clarity of the latter is vastly to be preferred to the obscurity of the former. Wittgenstein himself once said 'philosophy ought really to be written only as a poetic composition.' So he wrote in aphorisms, as did Nietzsche, another greater as a poet than as a philosopher.

As R. D. Laing has rightly claimed, schizophrenia and art can be closely intertwined (see, for example, the poet and artist Thomas Hennell's moving account of his own schizophrenic episode⁶). Thus one can read Wittgenstein or Nietzsche as poetry with benefit. But one cannot base a study of mind or perception on either. Anyone who thinks that Wittgenstein was a great, or even a good, philosopher should read Jenny Teichman's *Philosophy and the Mind* (chapter 4 and especially pages 50–51).

It is strange that many philosophers still believe that important problems relating to mind and perception can be solved by 'linguistic' or 'logical' analysis (also known as 'folk psychology') rather than by slogging it out with observation, hypothesis and experiment. It is amazing, for example, that so many philosophers still adhere to the pre-scientific theory — totally at variance with modern cognitive neuroscience — known as naive or direct (phenomenal) realism⁷.

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Physics lesson

SIR — John Maddox (*Nature* **354**, 263; 1991) missed the real lesson of the current state of physics. Attempts to attack the "grand questions" have led to such dead ends as Einstein's unified field theory, strings, the philosophy of quantum measurement and quantum cosmology. On the other hand, study of such apparently messy and prosaic problems as the thermodynamics of fluids near their critical points has yielded deep insights into the nature of the physical world. The lesson is that science is led by experiment, and that the proper job of theorists is to predict and explain the results of experiments. The research that strikes the editor as narrow at the least increases our understanding of the natural world in a small way, and may some day have greater implications.

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Misunderstanding

SIR — I was surprised to read Cesare Emiliani talking about miracles, God and the Devil in response to my letter about the Turin Shroud dating (*Nature* **352**, 187 and **353**, 598; 1991). I think I have been misunderstood. My purpose was not to "lament" anything but to ask this simple question: is there something inaccurate in the description of the Shroud samples provided in the report of Damon *et al.* (*Nature* **337**, 611–615; 1989)? One of the co-authors, Dr W. Woelfli, says "yes". If Woelfli is right, the error should be acknowledged in an authors' correction. Like many people, I believe that a careful description of the material and methods in a scientific report is a profitable approach. This is not a matter of God or Devil.

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ODSQ

SIR — In the belief that science might be better served by collections of quotations, we are compiling the *Oxford Dictionary of Scientific Quotations* to be published by Oxford University Press. We would welcome contributions, whether instructive, amusing, characteristic of a certain individual, crucial in formulating scientific principles, or whatever. Quotations may be from scientists both living and dead, and from other writers upon science. They may also be 'attrib.' or part of the 'folklore' of science. We would be especially grateful for slightly more out-of-the-way instances (perhaps quotations not yet famous but that deserve to become so).

W. F. BYNUM
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Maxwell's role

SIR — Your leading article about Mr Robert Maxwell (*Nature* **354**, 93; 1991) is less than fair to his achievements. First, you say that "many [of Pergamon's] journals are undistinguished", while acknowledging that "some" have excellent reputations. In fact, in almost every field of science, at least one Pergamon journal is among the world leaders; for example, *Neuroscience* (founded 1976) is rated 17th in terms of impact factor out of all the 4,300 journals in every field of science in the Institute of Scientific Information database.

Neuroscience illustrates another feature of Maxwell's scientific publishing: he published the journal but gave the ownership to the International Brain Research Organization (of which I am director of publications) most of whose income now comes from the journal. Many scientific societies have benefited from arrangements of this kind with Pergamon Press.

I was also sorry to see you quoting the Board of Trade report, without mentioning that a few years later several of the criticisms in that report were withdrawn by the Board of Trade as unfounded in fact.

Scientists throughout the world owe a debt of gratitude to Maxwell for his vision and for his long-standing support of their different disciplines. In all my dealings with him he was absolutely correct and always kept his word.

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Hanging out for the hypersonic plane

Roger Handberg, Joan Johnson-Freese and David C. Webb

The United States must learn how to cooperate with others if space research is to continue. The dilemma of the hypersonic plane is a case in point.

Now that the Cold War has ended, international cooperation in space research is a more attractive prospect for the United States. The costs of cooperative programmes, although higher in aggregate because of interface and coordination expenses, are spread across several participants, allowing each to pursue a broader mixture of national and international space projects than would otherwise be possible.

Recent US national efforts to develop a hypersonic single-stage-to-orbit launch vehicle provide an example to test whether there is a renewed emphasis on international space cooperation. Hypersonic is defined as a speed greater than mach 5 (five times the speed of sound). Although the space shuttle and expendable rockets fly hypersonically, we use the term 'hypersonic vehicle' to denote an aerospace plane that, like an aeroplane, takes off and lands horizontally, using advanced air-breathing engines to fly as high as possible in the atmosphere before switching to rockets. Obtaining oxygen from the air reduces the weight penalty associated with this fuel. The extraordinary engineering complexity and consequent expense of development have been significant factors limiting progress. Hypersonic vehicles operate at speeds up to mach 25, carrying up to a 25,000-lb payload into low-Earth orbit.

Why a hypersonic?

The US programme, the National Aerospace Plane Program (NASP), aims to build an air-breathing, single-stage-to-orbit vehicle using ramjets and scramjets up to mach 18 or above, shifting to a rocket system as the vehicle enters space¹. Ideally, such a system will increase access to space by allowing rapid turnaround using a small ground crew, thus operating at significantly lower cost than existing systems. Such a launch system would achieve many of the unachieved goals planned for the shuttle².

At least seven countries (the United States, the United Kingdom, Japan, Germany, France, Sweden and the former Soviet Union), alone or in some combination, are engaged in hypersonic development³. Traditionally, the United States has gone it alone in such advanced projects, especially those with obvious military potential⁴. This go-it-alone posture may, however, be less viable in a fiscal climate which is becoming in-

creasingly bleak and hostile to long-term space projects. The cost of moving one pound to orbit is at present too high to allow a flourishing programme of space exploration to be developed by any one country. Current estimates are that it costs between \$3,500 to \$7,000 per pound to achieve orbit, whereas the hypersonic holds the potential for lowering costs to between \$300 to \$1,000 per pound to orbit¹ (\$1,000 per pound to orbit is economically competitive).

Additional benefits will also accrue as a result of new technologies, including the development of new materials, advances in computational fluid dynamics and development of new propulsion systems. In this manner, both economic and scientific incentives are present in the development of a hypersonic vehicle. But the costs involved are extremely high: \$18,000 million–\$20,000 million for a single-stage vehicle, and even more for a two-stage concept. (The German Saenger-Horus combination is estimated to cost \$25,000 million.)

Cost sharing may realistically be the only way to develop hypersonic technology. British and Soviet groups are considering an innovative cooperative programme in which the Soviet Anton 225 transport aircraft would be the first stage for an all-rocket engine version of the British Hotol design. The Japanese have discussed hypersonic technology development with both the Germans and the French.

The United States has been more reluctant to cooperate in such efforts, in part because of concerns about military applications of the technology and because of the country's economic competitive position. Such standoffish attitudes may mean the hypersonic plane is never built, or is completed far too late.

Attitude, constitutional ability and planning capability have to be considered in examining US policy on international cooperation. Attitude is always a problem when military applications and economic competitiveness are concerns, but can be surmounted when sufficient benefits or overriding issues can be identified, for example the 1969 US Japanese space 'cooperation' agreement, which authorized the transfer of spacecraft and launcher technology by US aerospace companies to Japanese counterparts. This plan was approved, despite objections from NASA and the

Department of Defense, as part of a foreign-policy initiative to forge links between the United States and Japan. A more recent case is the FS-X aircraft coproduction agreement, allowing the Japanese to produce a next-generation aircraft based on the General Dynamics F-16 design⁵.

The US predicament

Both of these examples involve private-sector initiatives. The US government's ability to engage in international programmes is quite another story. From the outset, it must be acknowledged that the United States is not always perceived as a trustworthy international partner. For example, in 1979 the European Space Agency (ESA) and NASA agreed on a joint International Solar Polar Mission (ISPM) in which each agency agreed to build and fly a spacecraft to circle the polar regions of the Sun⁶. In 1981, the United States unilaterally cancelled its spacecraft due to budget reductions by the new presidential administration. Nevertheless, NASA upheld its agreement to launch the vehicle from the shuttle without cost and the ESA mission later flew as the Ulysses space project.

The ISPM cancellation without consultation created much unease among potential international space partners, evident particularly when the United States later solicited international participation in the Space Station Freedom programme. The Europeans demanded a guarantee from the United States that it was willing to commit the necessary funds to build and operate the Space Station. But there is no absolute guarantee that can be given to international partners in these circumstances because in the United States the allocation of funds for any project is carried out on an annual basis. The Europeans concluded that a treaty would be the obvious way forward, but this would have taken far too long. An intergovernmental agreement (IGA) has the status of a treaty, but in the United States an IGA cannot be upheld in the courts as the executive has no authority to bind Congress, or vice versa. About the best the Europeans could obtain were high-level, public statements supporting the Space Station to "enhance" the value of the IGA within the United States.

The international participants had, in

this case, somewhat greater leverage than those in the earlier ISPM because their financial contribution was critical in financing the Space Station. Hopes that the United States would be more considerate of the concerns of the international partners were dashed, however, when several redesigns of the Space Station were undertaken with no discussion of the significant budgetary consequences that followed for ESA, Canada and Japan.

The United States' inability to sustain international cooperative space efforts is a function of constitutional structure and domestic politics. International agreements and even treaties are not binding on subsequent budgetary decisions by the US Congress. Essentially, Congress takes up each budgetary item on a line-by-line basis each year, meaning that a programme can suffer dramatic charges in its budget in response to perceived needs in other areas. International programmes are thus subject to the vagaries of each year's budget cycle.

Such a system obviously works best when the economy is growing and sufficient money is available to complete most projects. In a period of economic stagnation or decline, competition between new projects is greatly accentuated. In addition, today space and other 'big science' projects such as the Superconducting Super Collider have become excessively complex and expensive, often resulting in projected cost being exceeded by factors of 50 to 100 per cent⁷. Such shortfalls are a combination of unforeseen technical problems and a strong tendency to underestimate real costs to get a project under way. The additional money is then sought later, a strategy that maximizes budget uncertainty over the long term. Further along in the process, the fiscal environment may change drastically, causing long-term programmes consistently to suffer stretch-outs and near-cancellations as newer schemes enter the pipeline.

Planning problems

Without the amendment to the Constitution, agreements between the United States and its international space partners will be complicated by the fact that the country cannot commit to multi-year projects beyond a certain level: a problem exacerbated by changeovers of presidential administrations. Space policy is not isolated from the system of successive administrations appointing their own people to positions deep within the bureaucratic agencies.

In the United States, planning (or the lack thereof) is another major issue affecting the possibility of international cooperative ventures. There is no central agency responsible for the direction of scientific and technical programmes. In-

stead, the formulation, adoption and direction of the country's efforts in these areas is left to the classic 'invisible hand' of *laissez faire* capitalism: the fittest survive. Such a maxim leads to the fiercest competitive battles between proponents of one programme over another; each is aided and abetted by the particular government agency having jurisdiction over the programme once it is funded. With 17 or more such bureaucratic juggernauts formulating their own plans and encouraging those from like-minded corporations, think-tanks, universities and non-profit organizations, titanic inter-agency battles often result. There are literally thousands of programmes being put forward at any one time. In recent years this entire process has been greatly accelerated by the fivefold increase in the number of congressional staff — many of whom spend their time formulating even more programmes. The result of this jungle approach — let it all hang out and the best will win — can be seen in the steady deterioration in the technical capabilities and position of the country. This slippery slope is littered with the corpses of once-funded programmes started in response to a perceived need, but later abandoned or stretched as other even more 'glitzy' projects move into view.

Why should the hypersonic programme be any different from other casualties of this system? Several factors suggest that the time may be particularly appropriate for change. First, NASA's role in the development of the NASP has been effectively stymied⁸. In the fiscal year 1992 budget, Congress reduced NASA funding for NASP to \$5 million. Meanwhile, the Department of Defense (actually the Air Force), the other main sponsor, sees NASP funding as a competitor to what it regards as more urgent projects, such as next-generation tactical fighters. Each year, the Department of Defense has attempted to reduce its support for NASP to use the money for other projects. Thus, the time is ripe for a consideration of alternative funding proposals, including international cooperative agreements, at the developmental stage of the project.

Another reason why the United States might now need seriously to consider new mechanisms for cooperative ventures is that construction of the space station will tax the shuttle fleet's lift capacity beyond its demonstrated ability to perform. The shuttle has a history of delayed launches, and such performance shortfalls become critical in constructing whatever version of the space station is finally to be built. The crisis will come if another vehicle is lost, and if space projects are then either stopped or severely limited. In addition, the shuttle

continues to be the most expensive means possible by which to gain access to space. Figures prepared by the Congressional Research Service show that the shuttle programme has cost \$63,700 million since its inception to the end of 1990, equivalent to \$1,670 million for each of the 38 flights completed during that time. NASA disputes these figures, saying that the total is \$42,000 million, still more than \$1,000 million per flight⁹. Such costs already severely hamper NASA's ability to do more than run the shuttle, a position that will be made worse by the Space Station, so that NASA may be able to do little significant scientific or industrial work¹⁰.

Where to now?

The United States is accused by potential international partners of dominating joint space efforts and being unwilling to allow others a significant voice in critical decisions. Earlier intransigence on the issue of control greatly facilitated the development of the European and Japanese space programmes: US policy on launchings was so restrictive and protective of economic and strategic interests that Europe moved to establish its independence so successfully that ESA and Japan are not far behind. Cooperative ventures may thus become more attractive to both sides: the effort becomes one of equals striving for a common goal, not a minuet dominated by one dancer. But this process will not be easy. Germany's reunification costs, the Soviet Union's disintegration, US budgetary problems and Japan's more modest space effort all suggest that a project as costly and complex as the development of an operational hypersonic air-breathing vehicle would best be undertaken internationally. Whether such a major venture in international cooperation would include the United States remains an open question. □

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Japanese industrial research

Small-scale industrial research in Japan stands out for its pursuit of excellence and for its willingness to devote time to fundamental investigations — if only to show customers what they might also achieve.

Tsukuba

WHAT makes Japanese high-technology industry so successful? What follows is inspired by curiosity about that question, but should not be mistaken for an answer to it. Rather, it is primarily an account of a visit to a handful of research laboratories at this "science city" ninety minutes by bus from Tokyo, but so different from it (or from any other Japanese city), with parks, four-lane highways near the centre and buildings with a room for a little landscaping around them that it is possible to believe the tale of a family that declined to move here because it preferred that its children should be brought up in a Japanese environment.

Tsukuba has changed a lot, and quickly. It was conceived, two decades ago, as a means of decanting research people and their families from Japan's crowded cities and downtown laboratories. At the beginning, it is said, there were people who preferred to commute every day rather than to abandon their familiar haunts in the biggest city. But now, the city has a university of its own, what some among the 250,000 residents say is a thriving cultural life and, collectively, such a strong thread of intellectual ambition that Tsukuba's public schools have become among the best in Japan — yet parents still send their children off (by bus, for the day) to even more highly regarded schools elsewhere.

On this particular visit, the reasons for visiting Tsukuba proved in the end to be spurious. Dr Chikara Hayashi, the physicist chairman of the high-vacuum company ULVAC, had mentioned at a party that there were two Chinese engineers working at his Tsukuba laboratory and several others at another at Chiba, on Tokyo Bay. Might they have something interesting to say about the state of Chinese research, China's relationship with Japan or even about the future of their government?

Hayashi had explained that the people concerned belonged to academic institutions at which he had made friends on his travels in China, and that they were proteges of friends of his. The two engineers, with the family names of Wang and Chang, are from Peking and Shanghai. They like it in Japan, and believe it would be better if they could stay for three years rather than the two they have contracted for. ULVAC, in the person of laboratory director Dr Sonoko Tsukahara, agrees to the

extent of saying that much of the first year is spent learning Japanese.

But Wang and Chang, diminutive people in their thirties with eyes sparkling with intelligence, quickly undermined their potential uniqueness by explaining that there are some hundreds of Chinese students at Tsukuba University. But that may be significant. If Japan cannot increase the enrolment of young Japanese in university engineering and science courses, may it have to look to the mainland to sustain the torrent of innovation of the past few years?

Earlier, Hayashi had been philosophical on that issue, recounting arguments in which Chinese had assured him that China would be "ahead" in fifty years. He smiled and shrugged his shoulders at the recollection, implying that he did not think it would be that simple, but that if it were, he would be the first to wish the Chinese well. He acknowledges that there is weight in sheer numbers.

Meanwhile Hayashi's company is a creature that would not, now at least, exist elsewhere. Its business is to make and sell high-vacuum equipment (not simply the boxes designed to contain less than nothing, but the pumps that empty them). Evidently the demands of the electronics industry in the past few decades have been immense, sufficient to sustain ULVAC in a growth phase. But is it not dangerous to rely on a single kind of customer?

Of course. That, says Tsukahara, is why one of her laboratory's main tasks is to find new uses for high vacuum techniques. The first objective is to make the potential market grow by showing potential users that the company's advanced technology has something they had not thought of to offer them — how to make magneto-optical storage disks, to coat anything with almost anything or to make magnetic recording tape with high coercivity, for example. Customers pay for the cost of answering the questions they are obliged to ask and the laboratory recovers some of its own costs by selling technical reports expensively to others who may be interested.

The company's laboratory is one of those at which you must exchange your shoes for plastic sandals at the front door. Another is the research laboratory of Hamamatsu Photonics, on the same site, whose stock-in-trade is the detection of single photons. Building on early experience with photomultipliers, the company

now reckons to be able to play its tricks in any region of the spectrum that may be of interest, and to be able to beat most of its competitors in sensitivity.

So what does Hamamatsu show off in its research laboratories? A tank filled with seawater, with sea-urchins clinging to the sides. The company has developed a technique for doping the membranes of growing sea-urchin embryos with fluorescent dyes so that their shape can be instantly determined (in "real time") from the patterns of single photons they emit. With the combination of such sensitive dyes and detectors, you can follow the rupture of a single lysosome membrane on a television screen. Who knows where that may lead? That is evidently the calculation behind an instrument maker's surprising support of basic biology research. But that seems typical of Japan.

The lessons from these and other tales are quite simple. The simplest route to success is to be able to do something that others cannot, counting single photons for example; but if you manage something in that category, you must always be conscious that there will be others treading on your trail, seeking to be a little better. The second route is that you must follow when you know your skill is not unique, and that you can succeed only by being better than another; then you follow another's trail, but hope to be quicker on the journey. That, of course, is nothing but a description of some kind of market. Japan's distinctiveness is that the criteria of success are defined in technical language — a resolution, an intensity or a stability may give you the decisive edge.

Japan has thus become the society in which only the best is passable, and in which the second-best is second-rate. These are stiff criteria, and potentially enervating as well. That is why it is as well that a group of liberally-minded amused people such as Hayashi are still in the vanguard of innovation. Between them, they settled for Tsukuba at which to build what is called the Tsukuba Research Consortium, a kind of science park sustained by the willingness of its founders to belong. (There are also satellite members, who rent space, and foreign members, who pay \$1,500 a year for access to data and the weekly seminars). Whatever happens across the Japan (or China) Sea, there seems no limit to its potential.

John Maddox

The making of the ear

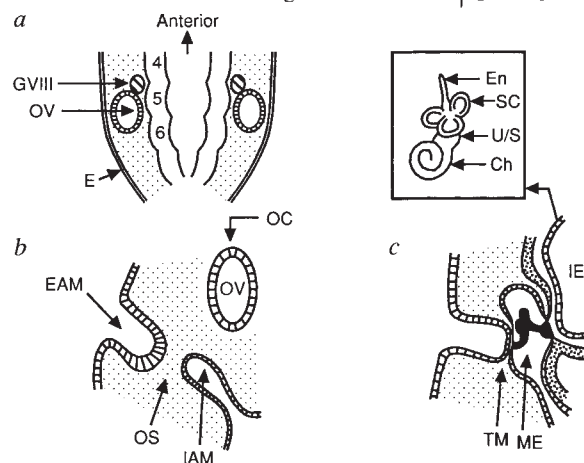
Brigid Hogan and Christopher Wright

Two laboratories, Pierre Chambon's in Strasbourg and Mario Capecchi's in Salt Lake City, have now used homologous recombination in embryonic stem cells to produce homozygous mice carrying a disruption in the homeobox gene *Hox-1.6*; the first group (Lufkin *et al.*) described their results last year in *Cell*¹, the second (Chisaka *et al.*) report theirs on page 516 of this issue². Once again the observations confirm the extraordinary power of this technique, otherwise known as gene targeting, for studying the genetics of mammalian embryogenesis. But determining precisely how the absence of *Hox-1.6* leads to serious defects in ear development and early postnatal death may be even more challenging than producing the mutants in the first place. Indeed, head development is so complex that only a concerted effort between embryologists, neurobiologists and molecular biologists will unravel the aetiology of the mutant phenotype.

The homeobox (*Hox*) genes regulate vertebrate development, and *Hox-1.6* is the most 3' gene of the *Hox 1* cluster. In early embryos, its messenger RNA is localized in both the neural tube and adjacent mesoderm, with a very anterior boundary near the junction between future rhombomeres 3 and 4 (refs 3, 4). Soon thereafter, expression retracts posteriorly, and becomes limited to a small region around the foregut. By alternative splicing, *Hox-1.6* produces transcripts encoding two related proteins, with and without a homeodomain⁵. Early on, both transcripts are equally abundant, but the one without a homeobox persists long after the other has disappeared³. The null alleles that were created disrupt either both transcripts (Strasbourg) or only the one containing the homeobox (Utah). Nevertheless, the two groups report very similar mutant phenotypes, except for some differences (of which more below). All homozygous mutants show defects in the hindbrain (neuroectoderm), the cranial nerves and ganglia (mainly neural crest), the inner ear (ectoderm) and certain skull bones (paraxial mesoderm). But, like *Hox-1.5*⁻ null mutants^{6,7}, *Hox-1.6*⁻ mice are affected in only the most anterior domains of gene expression, more posterior expressing tissues being unaffected.

The most dramatic abnormalities in the *Hox-1.6*⁻ mutant mice are in the inner ear, which forms only an inflated, thin-walled sac containing a few sensory cells. The normal morphogenesis of the inner ear involves a complex series of tissue interactions, only now being stud-

ied at the molecular level (see figure). It begins from an ectodermal thickening (placode) near the edge of the neural plate, at the level of future rhombomeres 5 and 6. By the time the placode has formed a closed vesicle, its spatial coordinates are probably well determined. Subsequent growth and differentiation of the vesicle depends crucially upon interactions with both the hindbrain and surrounding mesoderm^{8,9},



Development of the mouse ear. *a*, Coronal section through the hindbrain of a 9.5-day mouse embryo showing the otic vesicle (OV) adjacent to rhombomeres 5 and 6. Both the OV and the vestibulo-acoustic ganglion (GVIII) develop from a thickening of the ectoderm (E) known as the otic placode. *b*, Sagittal section through an older embryo showing the outer ear (external acoustic meatus, EAM) which develops from the first branchial cleft the internal acoustic meatus (IAM), which arises mainly from the first branchial pouch, and the rudiments of the three ossicles (OS). The incus and malleus develop from the neural crest-derived cartilage of the first arch and the stapes from the cartilage of the second arch. The OV induces a cartilaginous otic capsule (OC) in the adjacent mesenchyme. *c*, In later embryos the tympanic membrane (TM) is connected to the inner ear (IE) by the ossicles in the middle ear (ME). The OC has become ossified (dense stippling). The IE has become differentiated into a complex structure (inset) which now comprises semicircular canals (SC), utricle and saccule (U/S), cochlea (Ch) and endolymphatic duct (En).

both of which transiently express *Hox-1.6* in early embryos. The inner ear defects could therefore reflect either very early alterations in positional specification of the ectodermal placode, or a failure of trophic interactions with the hindbrain and/or mesoderm. Chisaka *et al.* report displacement of the mutant vesicle away from the hindbrain, so that short-range interactions may be disturbed. Whether this displacement is a primary or secondary effect is not known, but clues to that may come from the expression in the mutants of growth and transcription factors known to be expressed in and around the otic vesicle and hindbrain.

From the published reports, the ear

defects in the Utah mice seem to be more extensive than in the Strasbourg mutants, involving, in addition, both the outer and middle ear. In particular, the ossicles — malleus, incus and stapes — are missing. The first two are derived from first branchial arch cartilage, and the third from the second arch (see figure). Altered positional specification within the first arch, resulting in a failure to form the ossicles, would be particularly interesting, because *Hox-1.6* transcripts have not been detected this far anterior by *in situ* hybridization. Is it possible that more anterior expression, perhaps in only a few cells, has been

missed? A more detailed analysis of *Hox-1.6* expression in early embryos, using antibodies to detect both forms of the protein in individual cells, will certainly be helpful.

Besides the ear defects, both sets of mutants have serious abnormalities in the hindbrain between rhombomeres 4 and 7. Using antibodies to stain nerves in intact embryos, Chisaka *et al.* show that mutant posterior hindbrain ganglia, and associated nerves, are smaller, shifted anteriorly, and do not connect properly with the brain. Many of these structures develop from the neural crest, a population of cells that migrates from the neural tube after *Hox-1.6* RNA expression has retracted. This suggests that *Hox-1.6* specifies the developmental programme of neural crest cells or their precursors before they emerge.

Early postnatal death of the mutant pups is almost certainly related to these complex neurological defects. Many of them never

breathe, and it is known that respiration is controlled by hindbrain centres and cranial nerves which are perturbed in *Hox-1.6*⁻ mice. Some of the mutants generated in Utah, however, survive for as long as 3.5 days, and other differences in mutant phenotypes are observed between mice from the two laboratories. In many of the Strasbourg mutants, hindbrain neural-tube closure is delayed, something that is not observed by the other group. Perhaps more surprising is the apparent absence in the Utah embryos of the transient reiterated bulges (rhombomeres) that probably define functional compartments in the embryonic hindbrain.

What might cause such phenotypic

variability? Could it be related to variation in genetic background, or to the different targeting constructs used? Both groups used embryonic stem cells derived from the 129 mouse strain, and crossed their germ-line chimaeras to C57BL/6. However, extensive backcrossing is needed to put the mutation onto a uniform C57BL/6 background; until then, genetic variability will exist for both groups, which should ultimately provide important information about genes modifying development.

As described above, the Utah knock-out was designed to disrupt only the homeobox-containing transcript and should leave the other intact. Possible functions of homeodomain-less proteins have been proposed^{3,10}. But attempting to explain the differences in mutant phenotype on the basis of a role for *Hox-1.6* homeodomain-less proteins in embryonic development is premature, because we do not yet know whether the protein is normally made *in vivo*, let alone if it has a function.

The inner ear has been described as

"one of the most remarkable displays of precision microengineering in the vertebrate body"⁸. The *Hox-1.6*⁻ mutants will be invaluable for studying how it develops, as well as representing a milestone along the way to understanding craniofacial abnormalities and the basic organization of the nervous system. □

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MILKY WAY

Will the real Galactic Centre please stand up?

Paul T. P. Ho

IMAGES of the centre of our Galaxy, presented by A. Eckart *et al.* on page 526 of this issue¹, add to the mounting evidence that there is a black hole at the galactic hub. The infrared images, taken with the New Technology Telescope at the European Southern Observatory,

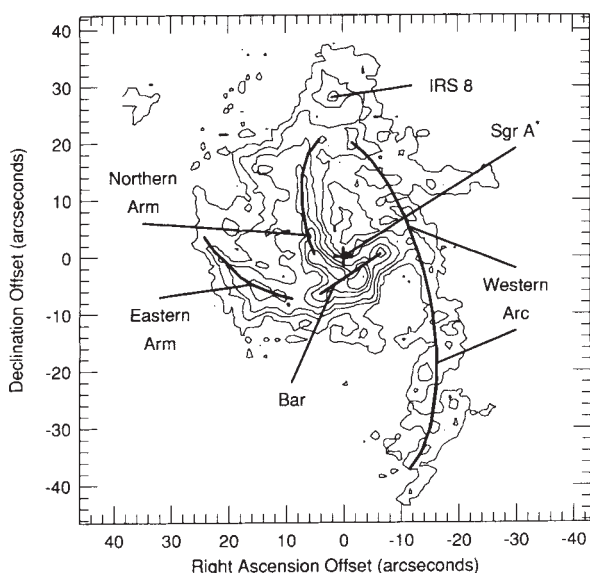
reveal about two dozen closely packed stars, a bubble of hot gas, and a compact source of infrared radiation that could be attributed to the action of a black hole.

The centre of the Milky Way lies some 8 kiloparsecs (kpc) from the Sun in the direction of the constellation of Sagittarius. Because of the heavy extinction by dust in the visual spectrum, the centre of our Galaxy can be seen only through infrared and radio techniques. In recent years it has been suggested, on the basis of gas dynamics, that a massive (2×10^6 solar-mass, $M_\odot$) black hole lies at the centre^{2,3}. If this were true it would be tremendously exciting, as our own Galactic Centre is probably at least 500 times closer to us than the next candidate for a massive black hole. Hence the best spatial resolution and the best intrinsic sensitivity can be achieved for this source. With the construction of the new Very Long Baseline Array by the US National Radio Astronomy Observa-

tory, angular resolutions better than a milliarcsecond can be achieved. At a distance of 8 kpc, the corresponding spatial resolution of 8 astronomical units (roughly the radius of Saturn's orbit) is about 200 times the Schwarzschild radius of a $2 \times 10^6 M_\odot$ black hole, from inside which neither matter nor light can escape. There have been two viable candidates for this black hole: the radio point source Sagittarius A* (refs 4, 5) and the infrared source IRS16 (ref. 6). If either one of these sources turn out to be a massive black hole, it would locate the true centre of the Milky Way.

Perhaps the best evidence for the existence of a compact massive object in the Galactic Centre comes from the study of the kinematics of the ionized gas streamers known as Sgr A West⁷. Recent studies³ of the Ne II (Ne^+) emission from these streamers show that if we interpret the motions as bound orbits, the deduced mass distribution cannot be satisfied with the known stellar density distribution (see figure). The point is that although several million solar masses are present in the form of stars, the mass distribution must be more centrally concentrated — a massive compact object is needed.

But which object is the purported black hole? The argument in favour of IRS16 rests with the early observations^{8,9} of helium recombination lines in the spectrum which show very broad emission-line widths corresponding to velocities of up to $1,000 \text{ km s}^{-1}$. If material moving at such speeds is in bound orbits around the source, a massive object must be at its centre. The new near-infrared observations by Eckart *et al.*¹ of the IRS16 complex resolve the source into about two dozen individual stellar sources, probably hot blue stars. These seem to add to the mounting evidence^{6,10,11} supporting the notion that the large emission-line widths are attributable to high-velocity winds from



Contours of emission from ionized neon (Ne II) from the Galactic Centre showing a one-armed 'keplerian' spiral, the western arc, orbiting Sagittarius A*. Lacy *et al.* argue that the gas velocities can be explained only if there is a $2 \times 10^6 M_\odot$ black hole at Sgr A*. (From ref. 3.)

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a number of blue supergiants.

The evidence pointing to Sgr A* as the host of a black hole, on the other hand, continues to hold up. The best radio continuum measurements show that Sgr A* is somewhat elliptical in shape with an intrinsic source size somewhere between 0.1 and 15 astronomical units (refs 12 and 13, respectively). Such a size and geometry could be consistent with an accretion disk around a massive black hole. The radio luminosity and size of Sgr A* are unlike those of any other source in the Galaxy. Perhaps the most persuasive and elegant argument in favour of Sgr A* remains the proper-motion experiment, which shows that Sgr A* is essentially at rest when measured against background quasars¹⁴. Although the upper limits to proper motions may be improved, the current values are consistent with the idea that being at the bottom of the gravitational

potential, Sgr A* will be stationary.

One lingering question on Sgr A* is also made somewhat clearer by Eckart *et al.* Until now, the absence of infrared emission from Sgr A* was troublesome. These authors now report the detection of a faint source at a wavelength of 2 μm . Furthermore, the faintness in the near-infrared may be related to a wind emanating from Sgr A* which appears to be blowing out a bubble of gas possibly as hot as 10^6 K, imaged by Eckart *et al.* in what they believe to be Fe XII emission. If this picture is correct, the wind may also be responsible for clearing out a cavity below Sgr A* revealed in the radio continuum emission, as well as for the wind-swept appearance of other radio continuum structures. $\square$

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TRANSMEMBRANE SIGNALLING

Pivots or pistons?

Andrew Pakula and Melvin Simon

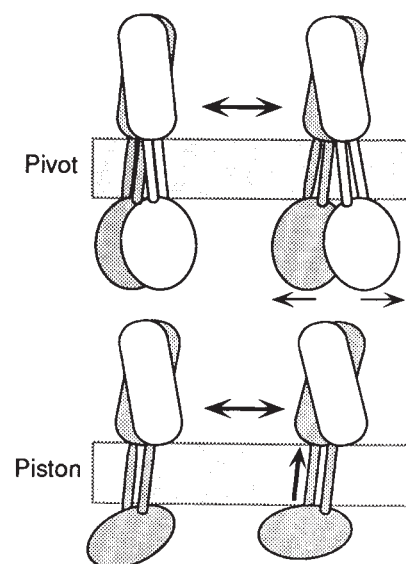
TRANSMEMBRANE receptors monitor the extracellular environment and transmit information about changes in specific ligand concentration to the cytoplasm. It is not clear how the message gets across the cell membrane, but one proposal is that ligand binding induces a transmembrane conformational change in the receptor. Writing in *Science*¹ late last year, members of Kim and Koshland's laboratories reported the determination of the three-dimensional structure of the extracellular domain of Tar, the bacterial aspartate receptor, with and without bound ligand, the hope being that the structures would provide a clear image of the conformational changes involved in signalling. Unfortunately, however, the picture remains blurred.

The bacterial chemotaxis receptor for aspartate bears a certain resemblance to mammalian receptors such as those for insulin and epidermal growth factor (EGF), in that they all have relatively large globular domains separated by a small number of transmembrane segments. Indeed, a chimaeric receptor composed of the periplasmic and transmembrane domains of the bacterial chemotaxis aspartate receptor fused to the cytoplasmic domain of the insulin receptor can transduce a signal in response to aspartate². Unlike the EGF receptor, however, the signal transduction mechanism of bacterial receptors does not seem to involve association and dissociation; the chemoreceptors are dimeric in both the presence and absence of ligand³.

Although attempts to crystallize intact

detergent-solubilized chemotaxis receptors have been unsuccessful, Milburn *et al.*¹ have succeeded in overproducing and crystallizing a fragment corresponding to the extracellular domain of Tar. They found that the introduction of a cystine crosslink facilitated the crystallization of this fragment. On the evidence of the observed structural differences between the aspartate-complexed and uncomplexed fragment structures, the authors propose that transmembrane signal transduction is mediated by a change in the orientation of the Tar monomers relative to one another, and not by structural changes within them. This conclusion, however, is contradicted by a more recent observation by Milligan and Koshland⁴, which implies that signal transduction occurs within individual subunits.

The three-dimensional structure of the Tar periplasmic domain reveals a dimer of two elongated four-helix bundles¹. Only very slight differences are seen between individual subunit structures when the apo and aspartate complexes are compared. The relative orientation of these subunits, however, changes by a 4° rotation about an axis that is roughly perpendicular to the dimer interface. Because the 'pivot point' is a considerable distance from the cytoplasmic domains, this small rotation might result in a rather large change in the relative positions of the cytoplasmic domains. Furthermore, as Milburn *et al.* point out, the intermolecular disulphide crosslink introduced into the protein to facilitate crystallization may have restricted what



Alternative models for the conformational changes that mediate transmembrane signal transduction. In the pivot model, monomers move only in relation to one another. In the piston model, structural changes occur within the individual monomers. As indicated, the piston model does not require both monomers to be intact.

would otherwise be an even larger motion.

Signalling by the Tar receptor causes a chemotactic response by modulation of the activity of a specific soluble protein kinase over a range of 2 to 300-fold⁵. As part of the adaptation process, signalling also causes the receptor to be methylated two to five times more rapidly by the CheR methyltransferase⁶. Milligan and Koshland⁴ prepared cystine cross-linked heterodimers between intact and truncated Tar receptors (see figure). Surprisingly, with a mixed dimer of intact Tar and a receptor fragment representing just the extracellular domain, receptor methylation is stimulated in response to aspartate binding, although the basal level of methylation is reduced about fivefold. This observation argues against models for signal transduction that require intersubunit transmembrane or cytoplasmic domain interactions. Milligan and Koshland suggest instead a class of models in which a signal is transmitted across the bilayer by motions of transmembrane helices in relation to one another within the individual subunits of the dimer. These might involve a variety of rotations and lateral or piston-like translations of transmembrane helices.

Other evidence supporting the notion that methylation signalling does not require a change in the relative positions of the Tar monomers has been presented by Lynch and Koshland⁷. In this work, it is demonstrated that an intermolecular disulphide linkage mediated by a cysteine substitution of transmembrane res-

idue 18 does not prevent the modulation of methylation rate by aspartate binding. It seems likely that such a crosslink would constrain any large intermonomer conformational changes; and if the motions were involved in the alteration of methylation rate, they would have measurable effects on this measure of receptor function.

What might be the basis of the apparent contradiction between the biochemical and crystallographic studies? First, the two solved structures may not actually be representative of the native structures of the apo and aspartate complex forms of the periplasmic domain in the intact receptor. For example, both structures may be like one of two alternative conformations, and the observed differences between the complexed and apo structures could be due to the effects of different crystal packing forces. Several factors might have contributed to such an occurrence, including the presence of the non-native cystine 36 disulphide crosslink which may have altered one or both of the structures. Formation of this crosslink has, in fact, been shown to mimic the effect of aspartate binding⁸. Furthermore, the complexed structure is unusual in that only one of the two possible aspartate binding sites is occupied.

A second possibility is that the structures do accurately represent the native uncomplexed and complexed receptor states. If this is the case, we might question the idea that methylation rate accurately reflects receptor signalling. It could be that the observed relative rotation of the subunits is the primary mechanism of signal transduction, and that intramolecular structural shifts that account for changes in methylation rate may be present in the available structures, but are too subtle to detect.

The new crystallographic data provide us with an elegant picture of how ligand binds to the dimeric aspartate receptor. But it will be a long haul yet before we fully understand the dynamic structural changes that mediate transmembrane signalling. □

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Function for Ras in sight

Anne J. Ridley and Alan Hall

SINCE the *ras* genes were first identified as human oncogenes a decade ago, finding the function of the Ras proteins has been the holy grail. As a result, the pivotal role of these GTP-binding proteins in regulating a variety of biological processes is no longer in doubt, but the way in which they work still remains a mystery. Now, using a genetic approach, Rubin and colleagues on page 559 of this issue¹, and in a paper recently published in *Cell*², provide evidence that Ras regulates cell fate determination in the *Drosophila* eye; more importantly, further analysis of this system should soon provide insight into how it does so.

Cell lineage

The *Drosophila* compound eye consists of about 800 repeating units called ommatidia, each of which has eight photoreceptor cells and 12 accessory cells. The relative ease of scoring mutations affecting eye development makes this an attractive system to define genes controlling cell lineage determination. Sixteen years ago, *sevenless* (*sev*) was identified as a gene essential for the normal development of one particular photoreceptor, the R7 cell. It turned out to encode a transmembrane receptor tyrosine kinase expressed by several cell types, including R7 cells³. Further analysis of mutations preventing normal R7 development led to the identification of a ligand for *Sev*, the *bride-of-sevenless* (*boss*) gene product, which is expressed on the surface of adjacent R8 cells⁴. It seems that *Boss* activates *Sev*, stimulating a signal-transduction pathway leading to the differentiation of R7 precursor cells into photoreceptor cells.

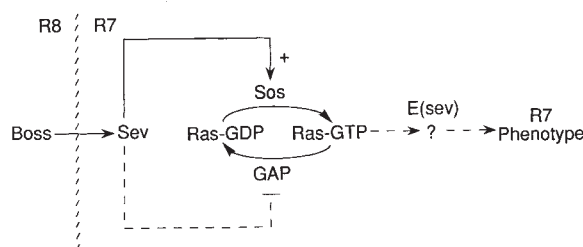
Identifying the players in the signal-transduction pathway downstream of the *Sev* receptor has proved more difficult, probably because they are involved in a multitude of other essential regulatory pathways, and homozygous inactivation of their genes would be expected to be lethal. To overcome this problem, Simon *et al.*² devised an elegant selection procedure, using flies expressing a temperature-sensitive mutant of *Sev*.

They reasoned that at a temperature where *Sev* is just active, mutation of only one of the two alleles encoding a downstream protein could reduce signalling to below the threshold required for R7 differentiation, while leaving *Sev*-independent signalling pathways intact. After screening 30,000 mutagenized flies for the absence of R7 cells, they identified mutations at seven distinct loci called *Enhancers of sevenless* (*E(sev)*), which decrease *Sev*-dependent signalling.

Given the widespread involvement of Ras in signal transduction, it was perhaps not too surprising that one of the *E(sev)* loci turned out to be the *Drosophila ras1* gene. A second locus mapped to a gene, *Son of sevenless* (*Sos*), that had already been shown to affect *Sev* signalling⁵. Simon *et al.*² now report that *Sos* encodes a protein with significant homology to CDC25, a guanine-nucleotide exchange factor for *Saccharomyces cerevisiae* RAS proteins.

These results imply that *Ras1* is an essential component of the signal transduction pathway activated by *Sev* in R7 precursor cells. To substantiate this hypothesis, Fortini *et al.*¹ go on to show that expression of an activated *Ras1*^{Val12} protein, specifically in those cells that normally express *Sev*, is sufficient to induce R7 differentiation in the absence of functional *Sev* or of *Boss*. Indeed, it leads to the formation of supernumerary R7 cells, derived from non-neuronal cell precursors. This story closely resembles that described in *Caenorhabditis elegans*, where the *let-23* gene encoding a receptor tyrosine kinase, and the *let-60* gene encoding a Ras protein, are required for normal vulval development, and where activated *ras* alleles induce a multivulva phenotype^{6,7}.

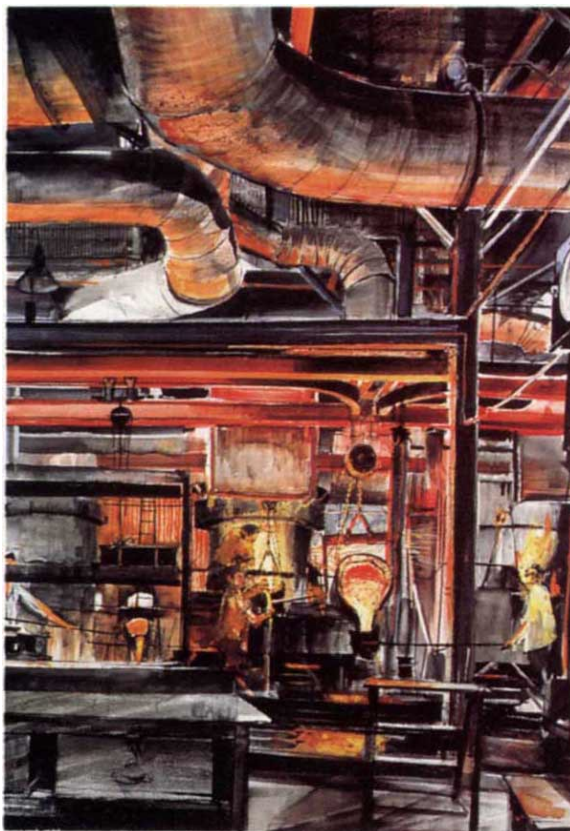
Interestingly, two other Ras-related proteins seem to be involved in eye development. First, an activated *ras2* gene, although 50 per cent homologous to *ras1*, induces a different abnormal phenotype of the eye, and is unable to replace the requirement for *Sev*¹. Second, the dominant eye mutation *roughened*, which prevents R7 development in about 50 per cent of ommatidia, has been mapped to a point mutation in a *Drosophila* homologue of the human *rap1* gene⁸. This suggests that the mutated *Rap1* somehow antagonizes normal *Ras1* function in the presumptive R7 cell, and is



A model for *Sev*-induced R7 differentiation.

Industrial heritage celebrated in art

ALTHOUGH science and technology are not generally thought to mix well with art, there are plenty of examples to prove the contrary: J. M. W. Turner's landscape *Rain, Steam and Speed*, a depiction of the Great Western Railway, is just one outstanding case. And Joseph Wright, a friend of Erasmus Darwin and Richard Arkwright (inventor of the spinning jenny) among others, is famous for his tableaux depicting bizarre scientific demonstrations from the eighteenth century. The Science Museum in London has opened a gallery in its precincts especially for the purpose of exhibiting industrial and scientific works of art, including its own permanent collection which spans 200 years. The gallery opens with a retrospective exhibition of the British industrial artist Edna Lumb, whose picture *Beams Foundry, Tipton* (1979) is shown here. Born in Leeds, northeast England, in 1931, Lumb has made a speciality of industrial scenes. Foreswearing the common tendency to regard industry as something ugly, she argues that "Mill chimneys, pit heads and quarries can be seen as so many sentinels giving the same elegance of composition in Yorkshire and Lancashire as poplar trees lining the roads in France. Until people see an exciting artistic interpretation of industrial landscape they may remain completely blind to its grandeur." In speaking also of the hypnotic power of machinery movement and the musical rhythm of the steam engine, she echoes the Italian Futurists and American Vorticists of the early twentieth century who sought inspiration in the dynamism of modern machine-dominated society. The Edna Lumb retrospective continues until 4 May. **R.P.**



analogous to the reversion of the *ras*-transformed phenotype in fibroblasts by overexpression of *Rap1a* (ref. 9). Little is known about the normal functions of either *Rap1* or *Ras2* (a homologue of mammalian *R-ras*) in any system, so further genetic analysis of their effects on eye development could well prove productive.

The simplest model to include all the genes so far characterized on the R7 determination pathway is shown in the figure. Boss, expressed on the surface of R8 cells, activates *Sev* on adjacent R7 precursor cells. Subsequent activation of *Sos* catalyses the conversion of *Ras1*-GDP to *Ras1*-GTP, which then initiates the downstream signals necessary to induce R7-specific differentiation. Establishing a link between *Sev* and *Sos* will require biochemical investigation, but

one possibility is that *Sev* could stimulate *Sos* activity directly by tyrosine phosphorylation. In addition, if *Sos* is proved to be an exchange factor for *Ras1*, then its isolation as an *E(sev)* locus would imply that nucleotide exchange on *Ras1* is a rate-limiting step in this signalling process. This is in contrast to a model for *Ras* activation in T cells, where the level of *Ras*-GTP is believed to be controlled by receptor-mediated inhibition of a GTPase-activating protein (GAP) (see figure)¹⁰. It is quite possible that either or both of these mechanisms can be used to regulate *Ras*-GTP levels under different circumstances.

Although the model in the figure is consistent with the data, studies on receptor tyrosine kinases in mammalian cells suggest that the *Sev* signalling pathway is probably not so straightforward.

First, some tyrosine kinases have been shown to act on several targets, including phospholipase *Cy*, phosphatidylinositol 3-kinase, *Raf* and *RasGAP* (ref. 11). It would therefore be surprising if *Ras1* were the sole mediator of *Sev* signalling; although expression of *Ras1*^{Vall2} can bypass the requirement for *Sev*, it may do so indirectly by leading to the production of signals not normally activated through endogenous *Ras1*. Second, stimulation of two different transmembrane receptors is often required for an optimal cellular response, for example in mitogenesis, and a model where *Ras* and *Sev* act on parallel pathways cannot be ruled out. The sensitivity of *Sev*-mediated signalling to changes in *Sos* levels could still be explained if *Sos* and *Ras* were regulatory components of a pathway acting synergistically with *Sev*.

Downstream targets

These complexities aside, the importance of the work of Rubin and colleagues is that they have identified *Ras* by genetic means as a crucial component of a receptor-mediated signal transduction pathway in development, and that this system is amenable to further genetic analysis. The next step is to define other components of the pathway downstream of *Ras*. The method of isolating the *E(sev)* mutations is unlikely to pick up downstream targets that are normally expressed in excess, and for which a twofold reduction in activity would not be rate-limiting. But there are already five other *E(sev)* loci to investigate, which could include an effector molecule directly regulated by *Ras*. It will be particularly interesting to see whether any of these genes share homology with the two characterized GTPase-activating proteins for mammalian *Ras*, *RasGAP* and *NF1*, as this could resolve the continuing controversy of whether these proteins are downregulators or the true effectors of *Ras* function. □

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Squeezing gas through space

Paul J. Wiita

DIRECTED flows are ubiquitous in astronomy. It has been recognized for over fifteen years that large radio sources outside our Galaxy are fed by jets emanating from the nuclei of active galaxies. The radio hotspots and lobes, understood to form where the jets interact with ambient gas, often extend to distances an order of magnitude greater than the sizes of the parent galaxies. For over a decade, collimated outflows have also been detected from protostars or young stellar objects. Planetary nebulae, the glowing ejecta from low-mass stars in late stages of their lives, also frequently exhibit cylindrical, rather than spherical, symmetry. Key questions about astrophysical jets are: can a single mechanism account for jet production on such widely disparate length scales, spanning a factor of 10^8 – 10^{11} ? And, if so, is the collimation essentially hydrodynamical, or do magnetic fields play a dominant role? In a paper emerging from research on planetary nebulae, Icke *et al.*, on page 524 of this issue¹, propose a rather simple mechanism using inertial confinement, which, they argue, can answer the first question in the affirmative and the second in favour of pure hydrodynamics.

Early models for extragalactic radio jets² did posit hydrodynamical collimation. An initially spherical flow of hot, low-density gas was assumed to be spewing from a central engine; however, the cavity it inflated would elongate as it forced its way out of a spheroidal (presumably rotating) cloud along the cloud's axis of symmetry. After carving out nozzles on each side, most of the fluid's initially random thermal motion would be converted to bulk kinetic energy, yielding a pair of oppositely directed jets which would plough outwards through an external plasma. Shock waves would then decelerate the fluid at the ends of the jets, converting bulk motion back into thermal energy, compressing magnetic fields, and accelerating electrons to relativistic speeds. These shock acceleration regions would emit a tremendous amount of radio synchrotron emission and would thus form the radio hotspots.

This basic picture did explain much about the structure of radio galaxies and quasars. In a scaled down version it could be applied to young stellar objects, where observations of asymmetric bipolar nebulae gave evidence of substantial mass loss in the form of outflows of molecular gas in opposite directions. It is now clear that a broad range of astrophysical phenomena, in-

cluding high-velocity H_2O masers, Herbig-Haro objects (compact dense clouds, identified with the termination of the stellar jets), shock-excited H_2 emission, and ionized interstellar jets, are all related to these directed outflows during the early stages of star formation³.

But several problems with such hydrodynamical models emerged, especially when they were applied to extragalactic sources whose jets often had opening angles of less than 1° . Both simplified⁴ and sophisticated⁵ two-dimensional hydrodynamic calculations showed that although interaction of hot plasma with a flattened confining cloud would indeed yield oppositely directed flows, these computed streams had substantially wider opening angles than were observed. Similar simulations did, however, make it clear that once narrow jets were established, they could remain thin without recourse to magnetic effects⁶. The pressures within the jets, particularly at the locations of bright radio knots (which have been identified with bi-conical shocks within the jet flows⁶), were often estimated to be much

greater than those of the external confining gas.

These and other arguments led to the development of a wide array of models relying mainly upon magnetic fields for jet acceleration and collimation^{7,8}. The magnetic fields were assumed to be generated within, and anchored to, disks of accreting gas around the central black holes or protostars. Over the past few years, the majority opinion has been in favour of *some* type of magnetohydrodynamical model for jet production and (at least initial) collimation, for both extragalactic⁹ and galactic¹⁰ jets. Such magnetized models are perforce complex, and usually very sensitive to detailed simplifying assumptions. So despite the promise of some recent analytical work⁸, there is nothing close to a consensus on which type of magnetic picture is best. Checking the consistency of all of these models has been nearly impossible, because simulating them requires high-resolution three-dimensional magnetohydrodynamical codes, which are still beyond even the fastest supercomputers.

Thus it would be quite exciting if it could be clearly demonstrated that a simpler, purely hydrodynamical mechanism was indeed capable of collimating jets on a wide range of scales. Icke *et al.*¹

IMAGE
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REASONS

Great and small. Left, radio emission from the bipolar nebula S106 (and a bright foreground source) in our Galaxy. The very faint source at the centre of the two lobes (which are separated by a dark band where there is essentially no emission) corresponds to a strong infrared source and indicates the location of the envelope of a young star. Material from the star emerges in two directions, and, interacting with external gas, creates the lobes of emission. Below, a radio map of the galaxy Hercules A, one of the brightest objects in the radio sky, clearly shows two jets emerging from a weak central radio source at the galactic core. These jets extend 4.5×10^5 light-years on either side of the central galaxy, but are clearly asymmetric: the eastern jet is continuous but exhibits wiggling instabilities while the western jet seems to break up into several apparently distinct rings.

have used a new two-dimensional computer code which verifies their analytical estimates that the morphologies of planetary nebulae can be understood in terms of an initially spherical wind interacting with a quasi-toroidal shell that is thicker along the equator. They find that narrow chimneys form along the symmetry axis of the toroid which provides inertial confinement. Gas flowing back from the head of the outflow exerts a substantial additional confining pressure. Also, as described in earlier analytical work¹¹, an inner shock forms with a barrel shape; this shock deflects the gas heading in the general direction of the toroid's equator up along the outside of the jet. The jets formed by this combination of hydrodynamical effects achieve high Mach numbers (their velocities far exceed the speed of sound) and are much less dense than the surrounding gas. They seem to scale in fashions capable of producing morphologies typical of radio galaxies and young stellar objects as well as planetary nebulae.

It is not yet clear if this unifying mechanism works well enough with relativistic fluids to yield the velocities extremely close to the speed of light that have been inferred to exist in some radio galaxy and quasar cores. Magnetic models may perform better in yielding very-high-velocity jets. These new simulations should be improved by using finer grids and allowing the jets to propagate to greater distances to check that the jets remain stable and narrow. The computations must be expanded from two to three dimensions to see if non-axisymmetric instabilities can spoil the pretty picture. Finally, magnetic fields, even if weak, are definitely present, and should be incorporated in these simulations. Only then will it be possible to confront the observational data in depth by deriving approximate radio maps of the sources from the numerical models. □

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A bent for charge separation

Marye Anne Fox

A FLEXIBLE molecule that snaps shut following illumination to make an almost closed loop has been devised by a collaboration of Dutch chemists led by Jan Verhoeven and John Warman¹. The molecule is the product of an attempt to stabilize the charge-separated species that often form upon excitation of light-sensitive compounds.

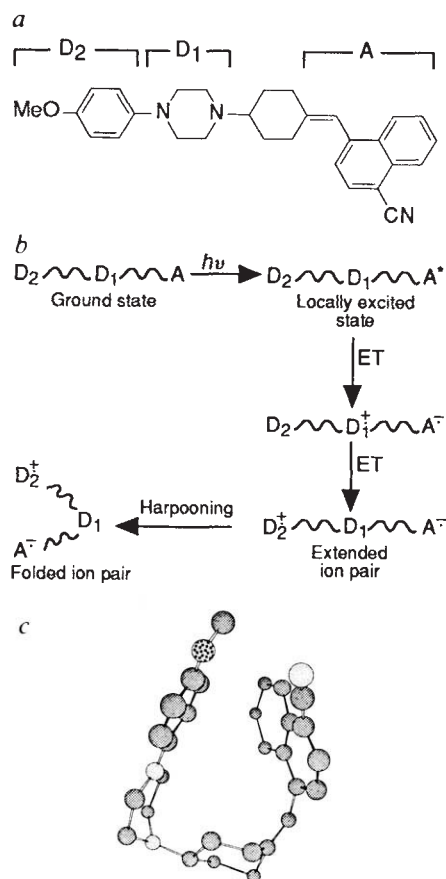
The formation of charged intermediate species following photoinduced charge transfer is often the first step in light-based technologies — photonics, molecular electronics, lithography and optical imaging. As in natural photosynthesis, subsequent chemical reactions are initiated only when the positively and negatively charged centres are generated at spatially distinct sites. Typically, the irradiated neutral molecule bears both an electron-rich (a donor) and an electron-poor (an acceptor) centre; on excitation by the absorption of a photon, charge is transferred from the

donor, which becomes a cation radical, to the acceptor, which becomes an anion radical. The presence of two opposite charges within a single molecule not only causes a profound increase in the molecular dipole moment (a pre-requisite for many sensor applications) but also increases the net conductivity of the medium containing the compound.

To exploit fully the chemical consequence of this charge separation, one must be able to control this switching from neutral to dipolar states by incorporating a correlated structural change induced specifically by the photoexcitation. This is the new achievement of the Dutch research groups, whose probe molecule (see figure) contains two carefully arranged donors and an acceptor and is sufficiently rigid to exist in an extended conformation in the neutral ground state. Upon photoexcitation, charge separation occurs to give a charged pair in an extended conformation. The electrostatic attraction between these opposite charges pulls the ends together, folding the molecule into a structurally distinct pair over a period of 10 nanoseconds or less. Because the folding reduces the distance separating the charged centres, it lessens the magnitude of the induced dipole. This conformational change, which was predicted from geometry-optimized semi-empirical calculations, was confirmed by nuclear magnetic resonance studies of the molecular ground state, by the observation of three distinct interconverting fluorescence bands following excitation (assigned respectively to the locally excited state, the extended charge-transfer ion pair and folded charge-transfer ion pair), and by time-resolved microwave conductivity measurements.

Because these two pairs are formed sequentially, this conformational change is accompanied by a time-dependent switching of the magnitude of the medium's conductivity. Verhoeven *et al.*¹ refer to this conformational change, which results from fast intramolecular electron transfer, as 'harpooning', a process in which the charged centres are drawn together just as a harpooned whale is drawn to a whaling ship.

Intramolecular electron transfer stimulated by light absorption has certainly been studied before by many groups². That research was stimulated by the elegant work by Calcaterra, Closs and Miller^{3,4}, which demonstrated that the rate of long-range, through-bond electron transfer across rigid hydrocarbon bridges is sensitive to the amount of energy released in the transfer, as had



a, The probe molecule of Verhoeven *et al.*, designed to demonstrate photoinduced harpooning. (D₁, D₂, two electron-donor moieties, of which D₂ has the less positive oxidation potential; A, an acceptor moiety.) b, Sequence of events leading to folding (*hν*, photoexcitation; CT, charge transfer). c, Molecular model of folded state.

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been predicted almost three decades earlier by Marcus⁵. Others then synthesized and characterized even more complex systems, for example, bearing three or more organic donors and acceptors on a common covalent frame⁶, or an organic donor tethered to an inorganic cluster⁷ with backbones varying in physical character from rigid to flexible. The critical role of the precursor conformation in exciplex formation has also been demonstrated previously, for example, in van der Auweraer, Gilbert and de Schryver's study of alkanes of varying lengths capped by donors and acceptors⁸. What is novel about the work of Verhoeven *et al.* is its clear demonstration of conformational change induced specifically by the intramolecular electron transfer.

For this electrostatically driven folding to follow light-induced electron transfer, several criteria must be met: first, an energetic driving force sufficiently strong to cause light-initiated electron transfer over large distances from the donor to the acceptor moiety; second, dispersion of the molecule of interest within a nonpolar solvent where solvation of the directly formed charge-transfer state is BIOGEOGRAPHY

less stabilizing than the coulombic attraction between the charged sites; and third, a molecular backbone sufficiently rigid to be fully stretched in its preferred ground-state conformation, yet maintaining enough flexibility to fold in response to the interaction of the opposite charges. Although these criteria may not be applicable to all cases of conformational change, they may be relevant in other systems. For example, conformational gating of ion transport in proteins⁹ may be responsive to many of the same factors. □

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Enemies doomed to associate

Jared M. Diamond

TAKE two species. If, when their distributions are mapped in chessboard fashion, they appear on different squares only, one might conclude that they are competitors; and if they consistently coexist on the same squares, one might think they are mutualists. But things are not so simple, because such patterns might well arise from the species' differing or coincident preferences for a particular habitat. That problem, for a long time a thorny one for ecological biogeographers, has been tackled by T. Schoener and G. Adler (*Am. Nat.* **137**, 669–692; 1991), who have now come up with a highly effective way of separating the effects of species interactions on species distributions from the effects of habitat variables.

The distinction matters especially in studies of guilds of potentially competing species. Precisely because competitors must be ecologically similar, their shared habitat requirements lead indirectly towards coincident distributions — the opposite of the direct effect of competition itself, which may lead towards complementary distributions. For example, two competing species of freshwater duck will both tend to be absent on islands without fresh water but present on islands with fresh water. Not surprisingly, most statistically significant associations among bird species of the Bis-

marck archipelago are positive, reflecting the familiar overwhelming effect of habitat requirements (M. Gilpin and J. Diamond, *Oecologia* **52**, 75–84; 1982). One would therefore expect that, if this dominant trend towards positive associations due to shared habitat requirements could somehow be stripped away, stronger evidence of negative associations due to competition would be unmasked.

Schoener and Adler's technique combines two elements. The independent variables are 16 continuously varying ecological parameters likely to influence species distributions, such as island area, isolation, maximum altitude, habitat or foliage diversity, and amounts of vegetation at various heights above the ground. The dependent variables are species codistributions, expressed in multiway contingency tables of the presence or absence of particular species (for example whether a given island supports species A and B, A but not B, B but not A, or neither). The database used for the test consists of published distributions of 40 species of diurnal lizards and resident birds on 52 Bahamian islands. In practice, Schoener and Adler construct two-, three- and four-way contingency tables (examining associations within sets of two to four species) for 23 guilds of lizard or bird species selected as being ecologically homogeneous (ground

Hopalong

ERRATIC hopping movements of water droplets on the warmed surface of solid cyclohexane provide much-sought evidence of microscopic forces at interfaces, report A. Steyer *et al.* (*Phys. Rev. Lett.* **68**, 64–66; 1992). The droplets were condensing from a stream of water-saturated nitrogen, and the surface was maintained just below 6.68 °C, cyclohexane's melting point. The authors were surprised to find the growing drops move, rotate and even jump. The explanation for these phenomena is in the forces created at a drop's edge by surface tension, which elastically deform the substrate structure. The heat of condensation released as the drop grows is just enough to melt the solid locally, allowing the elastic strain to be released in a quick flick. If a drop is pinned to a surface defect, it is likely to spin rather than jump. Until now, these contact forces had been talked about, but had not been observed on rigid surfaces.

Head gear

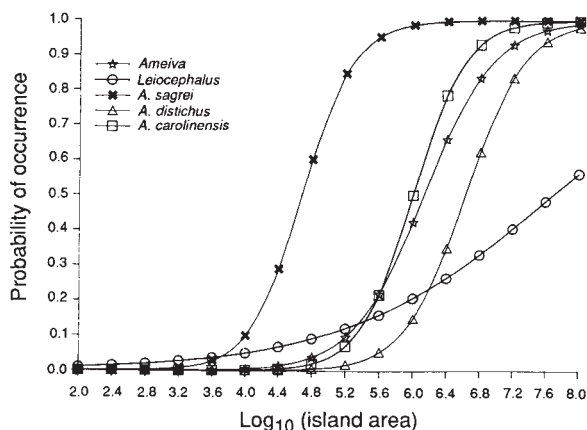
THE single-headed version of myosin, myosin I, is known to bind directly to lipid molecules, unlike the more common double-headed myosin II. T. D. Pollard and colleagues (*J. Cell Biol.* **116**, 367–376; 1992) have used the gliding filament assay to demonstrate that myosin I, when bound to pure lipid bilayers, is able to activate the movement of actin filaments. The new results provide an explanation for how myosin I can support motility of certain cells and organelles, and they suggest a mechanism for how the molecule is targeted to plasma membranes in *Acanthamoeba*, *Dictyostelium* and some vertebrate epithelial cells.

Milky ways

THE well-known but poorly documented advantages of mothers' milk are given a firm basis in a UK study reported in this week's *Lancet* (**339**, 261–264; 1992). A. Lucas and collaborators show that preterm babies whose mother provided breast milk (either by breastfeeding or by a tube) for the early weeks of life had a substantial advantage in subsequent IQ at 7–8 years of age. The authors adjusted for various social and economic factors, but genetic factors and differences in parental behaviour cannot be completely excluded. Lucas *et al.* believe that their data provide a strong indication that human breast milk contains one or more components that influence neurodevelopment. One of the important implications for infant care would be the reintroduction of milk banks, now generally fallen out of use in Britain, by which premature and other babies can be fed milk donated by lactating mothers rather than infant formula.

lizards, small insectivorous birds and seed-eating finches, for instance).

The first step in the analysis consists of logistic regression, for each species and independent variable, of that species' probability of occurrence on an island as a function of the variable. Such regressions are exemplified by graphs of 'incidence' (probability of occurrence) as a function of island area or species number. By an iterative procedure Schoener and Adler extract such species/habitat regressions plus the interactions within each combination of species (that is,



Logistic regression of the proportions of Bahamian islands in a given size class occupied by various lizard species, as a function of $\log_{10}$ -transformed island area. That proportion of islands has been termed 'incidence'. The steeper the rise of the curve, the sharper in some sense is the threshold range of island sizes separating islands with and without the named species. (From T. Schoener and G. Adler *Am. Nat.* **137**, 669–692; 1991.)

distributional overlap or avoidance not explained by the species/habitat regressions themselves). The authors stress that the application of this statistical technique to distributional data is new and warrants further testing.

The most striking conclusion is that, after correcting for the effects of habitat variables, the analysis tends to convert positive two-way associations between species (as observed in the raw distributional data) into negative associations. That is, although the direct interaction between the two species is actually negative (they tend to exclude each other), the interaction is masked in the raw data by the similar responses of both species to habitat variables. In effect, the species are enemies doomed to seek out the same types of sites. For instance, to return to the example of two competing duck species, both ducks would be absent from small islands without ponds, both would be present on islands large enough to contain different types of ponds, and only in the range of medium-sized islands with just a single pond would the ducks occupy different islands to the exclusion of each other.

These masking effects of habitat variables prove to be huge. As a result of

stripping them away, the proportion of two-way species interactions that are negative increases from 24 to 67 per cent for lizards, and from 3 to 47 per cent for birds. In addition, interactions that are already negative in the raw data become even more strongly so after considering habitat variables.

To interpret the signs of these interactions, Schoener and Adler note that the negative interactions are likely to represent competition, because the species analysed were chosen to be members of the same guild. Examples include well-

known pairs of species that have provided textbook instances of complementary island distributions, such as the mockingbirds *Mimus gundlachi* and *M. polyglottos* or the lizards *Anolis sagrei* and the *Leiocephalus* group. The residual positive interactions may represent parallel responses of species to habitat variables not among those analysed by Schoener and Adler. So two hummingbird species are positively associated both in the raw data and after correction for habitat variables — but probably because distributions of both species are tied to flower abundance, not among the dependent variables measured.

Several other points emerge from the study. Three-way interactions are overwhelmingly negative, reflecting in part the importance of diffuse competition. Negative interactions are more common among lizard species than among bird species, possibly owing to birds' higher immigration rates, hence smaller populations and shorter population lifetimes. Raw habitat variables yield stronger trends than do compound variables produced by principal components analysis.

The main message, however, is that if one wants to test occurrence data for species interactions, one should control for habitat relations because of their huge masking effect. Biogeographers will now be stimulated to reanalyse the many published lists of species occurrences on islands (as well as on island-like habitat patches) of known area, isolation and elevation. And field workers will be stimulated to measure a greater variety of habitat variables in their studies, for Schoener and Adler have now provided a recipe for taking such measurements into account □

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Occult learning

LAST week Daedalus proposed his 'Skygazer' space telescope. Its forty thousand photodetectors, dividing up the sky between them in fly's-eye fashion, will count all the photons hitting the telescope from the celestial sphere. With appropriate shielding from the Sun, Earth and Moon, it will be exquisitely, ultimately sensitive to the slightest variation of brightness from the rest of the sky.

The Skygazer will be ideal for locating stellar occultations. Daedalus estimates that in a galaxy of ten thousand million stars all wandering around, there must always be at least one star eclipsing another as seen from the Earth: yet (except in the special case of eclipsing binary stars) such an occultation has never been observed. This is not surprising. An occultation is quite undramatic, gives no warning, and cannot last much longer than a day. You would never spot it unless you knew where to look.

The Skygazer spacecraft will tell you. It watches all the sky all the time. Its ultimate photometric accuracy will instantly register the slight loss of light from that section of sky where one star is beginning to hide behind another. The detector whose count is changing will locate the region of loss to within one square degree of sky. An Earth-based Schmidt telescope could then be trained onto that region to find the occultation.

From the changing brightness and spectrum of this joint stellar image, useful information will be gained about the relative velocity, size, brightness, limb darkening and even shape of the protagonists. As the stars then separate, the shifting image of the occulted star as its light is deflected relativistically by the mass of the occulter, will give a measure of that mass. Occultations will be more frequent in some parts of the sky than in others, yielding statistical data on the density and velocity-distribution of stars in various regions of the galaxy.

Best of all, occultation offers about the only possible way of spotting the mysterious 'dark matter' that cosmologists insist must be lying about somewhere. Brown dwarfs, big planets, black clouds and general nonluminous celestial junk, will at last be detectable as they drift in front of luminous stars. If such dark objects have an atmosphere, or even a refractive index, informative lens-effects will further enrich their occultations. And should the long-feared impacting asteroid, or a spacecraft crammed with militant little green men, ever head menacingly towards the Earth, it should reveal itself by occulting background stars long before it could be detected on radar.

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Antarctic global warming?

SIR — Doake and Vaughan¹ report on the rapid disintegration of the Wordie Ice Shelf, Antarctica, using as evidence positions of the ice-shelf front in 1966 as mapped from aerial photography, and satellite images taken between 1974 and 1989. They attribute the disintegration of this floating mass of ice (from about 2,000 km² in 1966 to about 700 km² in 1989) to a warming trend in mean annual air temperature in Marguerite Bay, on the west side of the Antarctic Peninsula. Zwally², in an accompanying News and Views article, relates this observation to the question of the future stability of the West Antarctic ice sheet.

On a smaller scale, but also relevant to the results of Doake and Vaughan, are changes in a glacial feature at Stonington Island (68° 11' S, 67° W), about 65 nautical miles north of the Wordie Ice Shelf (69° 15' S, 67° 45' W). John Rymill, leader of the British Graham Land expedition (1934–37) established a camp on nearby Debenham islands, and was apparently the first to describe the snow ramp that connected Stonington Island to the mainland³. In 1939, the US Antarctic Service Expedition established East Base on the north end of Stonington Island, and also noted the “foot of a glacier which descended from the Antarctic Peninsula [that] tied Stonington Island to the mainland”⁴. FIDS (Falkland Islands Dependencies Survey) Base E was established in 1946, a year before the Ronne Antarctic Research expedition (1947–48) arrived at Stonington Island to reoccupy East Base. At this time, the snow ramp was still in place and connected the island to the mainland. Fraser⁵ noted the ramp in place in 1961–62, mapping its minimum width as 175 m and a gradient of near zero (sea level) to 30 m elevation in a horizontal distance of 100 m. The ramp was thus a massive feature, essentially an extension of Northeast Glacier, and was used by these expeditions for access from the island to the mainland during sledging trips.

In February 1975, the US Antarctic Research Program's research vessel *Hero* entered Marguerite Bay and visited Stonington Island. The snow ramp was noted by Lipps⁶ as being in place at the time of his visit. On 28 February 1990, I was on the cruise ship *Society Explorer*, which sailed into Marguerite Bay and anchored near Stonington Island. I noticed that the snow ramp connecting Northeast Glacier on the mainland of the Antarctic Peninsula and Stonington Island was no longer there, but there was instead an open-water channel between Back Bay and the main part of Marguerite Bay. This channel was about

50 m between the island and the vertical cliff face of Northeast Glacier, which was about 20 m high. When this snow ramp disintegrated and disappeared is unknown, although I made some attempts to establish the date as closely as possible.

C. Doake of the British Antarctic Survey provided a copy of a field diary from a British Antarctic Survey sledging team in July 1980, in which the ramp is described as having “a huge abyss separating ramp and glacier”, and “many large crevasses and seracs . . .”. Jane G. Ferrigno of the US Geological Survey assisted me by locating six usable scenes of the 54 Landsat pictures of the Stonington Island area between 1973 and 1990. She examined the March 1986 and December 1978 images in photo format, and she states in a letter to me that these two “images seemed to show no disintegration of the ice although the latter

image was partially obscured by cloud and shadow. The 1988 and 1989 images seemed to show a partial disintegration of the ice, but the microfiche was not adequate for accurate determination.” I have since discovered no further conclusive evidence.

Northeast Glacier is but one small part of the ice margin of numerous glaciers on the Antarctic Peninsula but it, together with observations by Doake and Vaughan¹ of the much larger Wordie Ice Shelf, can contribute to the effects of what might be a progressively warming period in this part of Antarctica, and subsequent retreat and disintegration of major ice shelves.

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Greenhouse indicators in Kenya

SIR — High mountain glaciers of the tropics are sensitive to climatic change. Mount Kenya's glaciers (9° 09' S, 37° 19' E) were largest in the late 1800s and have since receded drastically. The maximum extents are preserved by glacial moraines, while later stages are documented by expeditions to the mountain; all glaciers were mapped by photogrammetry in 1963 and again in 1987 (refs 1, 2). Earlier studies have indicated that the ice shrinkage during 1899–1963 was instigated by precipitation and temperature change, but essentially controlled by increased solar radiation absorption^{1,3}. The 1963 and 1987 mappings provided the basis for a quantitative evaluation of the climatic forcing of the observed ice changes over the past 25 years, particularly in the light of possible connections with the greenhouse effect.

In 1963–87 the total ice-covered area on Mount Kenya decreased by 40%, or at a considerably faster rate than during 1899–1963. Moreover, in contrast to 1899–1963, during 1963–87 the rate at which mass was being removed from the ice surface, termed surface lowering or thickness change, was essentially independent of solar radiation. Thus, processes other than solar radiation must have been the controlling factors of the ice wastage. Of particular interest are the energy effects on the ice of changing atmospheric composition and humidity, and environmental warming. The all-glacier mean ice thickness change from 1963 to 1987 amounted to –14.5 m, corresponding to –50 cm liquid water equivalent per year. The energy required

to dispose of this layer by melting is 5.4 Wm^{–2}.

Apart from energy considerations, decreased snowfall could also bring about a glacier shrinkage, but a precipitation decrease of the required large magnitude is not borne out by stations on the Kenya highlands. Similarly, enhanced solar radiation absorption resulting from changes in cloudiness, atmospheric turbidity, and glacier albedo related to increased dust deposition or less snowfall are all excluded, because these mechanisms would all lead to radiation-related differential thinning of the glaciers, which is not observed.

Therefore, the cause of the ice loss must be sought in changes in sensible and latent heat transfer and/or the net longwave radiation. We have examined the effect of small changes in environmental conditions on these energy forcings⁴, recognizing that the glaciers are in an elevation range of 4,600–5,000 m (580 mb), near the annual-mean 0 °C isothermal surface, and that the temperature of the glacier surface cannot rise above 0 °C. Particularly relevant to the latent heat forcing is the identification for the equatorial belt of a specific humidity increase of 0.6 g kg^{–1} in the layer 700–500 mb over 1965–85. This has been related to the global warming and greenhouse effect, and explained as a consequence of intensified sea–air interaction over the tropical oceans favoured by an increase in sea surface temperature⁵.

Based on a sensitivity analysis⁴, we find that the following combination of climatic forcings is the most plausible to

supply the energy for the observed ice thinning: a longwave radiational contribution from direct thermal trapping due to increasing greenhouse gases estimated from the literature to be $0.5\text{--}1.0\text{ W m}^{-2}$; a warming of $0.0\text{--}0.2\text{ }^{\circ}\text{C}$ contributing through sensible heat transfer $0.0\text{--}1.4\text{ W m}^{-2}$; and a $0.1\text{--}0.2\text{-g kg}^{-1}$ increase in specific humidity, in part induced by atmospheric warming, which through latent heat transfer would make available $2\text{--}4\text{ W m}^{-2}$.

The recent drastic wastage of Mount Kenya's glaciers indicates that significant climatic change is occurring in East Africa. In particular, the energy requirements for the observed ice loss point to the pivotal role of increased atmospheric water vapour content as part of a green-

house forcing. A new mapping of Mount Kenya's glaciers would serve to monitor the continuation of such climate controls.

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Garbage in paradise

SIR — The high Arctic is the “floor of creation”¹, the land having only recently emerged from its protective cradle of ice. In spring 1990, I was a member of a 1,200-km ski-traverse of Ellesmere Island ($76\text{--}84^{\circ}\text{ N}$), the northernmost land in North America². We came to realize that if curiosity in paradise was the original sin, then certainly the cavalier jettison of human garbage must be a close second.

Far from being an untainted wilderness, Ellesmere Island is riddled with the detritus of human passage and occupation. We encountered 208 objects, listed in the table, or one piece of garbage every 4 km of non-glaciated surface travelled. Yet Ellesmere Island is remarkably isolated, containing only about 100 permanent residents although it is the size of Great Britain.

No single problem of garbage is more pervasive in the Arctic than that of aircraft gasoline drums discarded by scientists studying the natural environment. A further irony is that the single largest collection of garbage we encountered was identified as belonging to a geologist who had written an article arguing against establishment of a national park on Ellesmere Island because it would attract too many tourists to an “environment [which] has exceptional limits in sustaining and suffering human use, even by the most modest southern standards”. The Polar Continental Shelf Project, the umbrella government organization that coordinates all research in the Canadian Arctic, must enforce a ‘garbage in – garbage out’ policy to all researchers relying upon its support.

The high Arctic is extremely unforgiving when it comes to absorbing human insults against its environmental integrity. The desert climate and harsh temperatures preserve our accumulative abuses. In an environment where

one can easily find relics of historic expeditions of centuries ago, the present waste will always stare back at us in mute testimonial to our arrogance. Items now left behind in the age of air travel can no longer be regarded as relics, just refuse.

HARVEST FROM ELLESMERE ISLAND

Gasoline drums (45-gallon, several stencilled “Eureka”)	47
Rusted metal food containers	41
Rifle cartridges	15
Gasoline drums (25-gallon)	14
Snowmobile parts	14
Self-aggrandizing cairns by Army personnel	10
Food crates (wooden and metal, each containing about 50 items)	7
Recent news magazines	6
Metal cans and cylinders	6
Cigarette butts	6
Angle iron (3-m lengths used to weigh down plastic for cartographic bench mark, 1 with stamp “1975”)	6
Pieces of clothing	5
Plastic garbage bags (empty)	5
Bales of wire	4
Scientific crates (some identified)	3
Snow gauges	3
Tarpaulins (plastic and cotton)	3
Tent heaters	2
Cotton and plastic cloth (15-m lengths)	2
Wooden boxes	2
Styrofoam drinking cups	2
Flare cannisters	2
Wooden tower (for radio antenna)	1
Graffiti (3-m letters for incoming helicopters spelling out sexual slang)	1
Surveying steel cable	1

This survey, together with that of Benton's for Ducie Atoll³, indicates that though some of the most remote corners of the globe may be technically classified as ‘wilderness’ through absence of human inhabitation, nowhere is the world really free of human waste.

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Prune function?

SIR — The sometimes surprising and unexpected results yielded by emergent similarities between products encoded by mutationally defined genes and other proteins with biochemically defined functions can sometimes lead to new insights into cellular function. Such has been proposed to be the case for the *Drosophila* eye-colour gene *prune* (*pn*), which Teng *et al.* state implicitly¹, and Ruggieri and McCormick explicitly², encodes a protein with significant similarity to GTPase-activating proteins (GAPs). Based on this conclusion, Teng *et al.* develop a model¹ where the *abnormal wing disks* (*awd*) gene product, a nucleoside diphosphate kinase principally associated with microtubules³, has a minor role in activating a hitherto unidentified Ras-like protein. The role of the *pn* gene product is then construed to be a down-regulator of this unidentified GTPase.

However, we believe that the *pn*/GAP similarity is far from significant. First, screening the PIR database (release 29.0) with the *pn* protein sequence using WORDSEARCH⁴, no outstanding scores are produced. However, the best ones that are produced are greater than that obtained against GAPs. The first of the GAP family of proteins (bovine GAP) is only the 102nd listed protein, there being 70 proteins with actual higher scores. It is therefore not clear how Teng *et al.* arrived at the conclusion of GAP similarity. A similar result was obtained using FASTA⁵ and BLAST⁶ searches, the last of which examines all protein databases and has a useful probability score which indicates that all of the matches are not significant (the best score, against acetyl CoA carboxylase, being 66, $P=0.2$).

Second, attempts to align *pn* and bovine GAP using ALIGN⁷, which includes a 100-fold randomization of the sequences to provide statistical meaning, shows that with gap penalties of +7 or +8 and with a mutational data matrix value adjustment of +5, alignments with numbers of gaps similar to those reported¹ are produced (21 and 19, respectively) yet the scores for the alignments lie within 0.5 s.d. of the mean of the randomized scores. This means that 30% of all randomized sequences score better and that therefore the sequences are unrelated.

Third, using the *pn* subsequence claimed to correspond to the most conserved sequence within the GAP catalytic domain (see Fig. 3d in ref. 1), we were unable to produce significant alignments to GAP proteins nor any significant matches to other protein sequences in the databases. Furthermore, using ALIGN on the *pn* subsequence and the

corresponding sequence from rap1 GAP, which appears superficially to be the most similar in this region to *pn* according to Fig. 3d of ref. 1, no significant alignment was produced; in fact neither did the rap1 GAP subsequence when compared to the Ras GAP subsequences (contrary to the implications of Fig. 3d). Indeed, it has also been the impression of others that rap1 GAP is not significantly similar to any other protein, either compared globally, or using this subsequence⁸.

Consequently, we find that there is no similarity between *pn* and GAPs — *pn* is as likely to be a GAP as is any randomly chosen protein. In the absence of confirmatory biochemical or genetic data concerning the model proposed by Teng *et al.*¹ must therefore be regarded currently as untenable. Because their model fails to explain the known biochemical defect in eye pigment production in *pn* flies^{9,10}, the simplest and not very unusual explanation would be that *pn* encodes a novel protein of unknown function.

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VENKATESH AND TENG REPLY — It is evident using computer comparison programs that the deduced TcD37 protein of *Drosophila* does not exhibit a high degree of homology with GAP or GAP-like proteins and might therefore be a novel protein with an unknown function. However, by these very same criteria (as noted by Barnes and Bürglin and others⁸), computer analysis would

not predict that mammalian rap1 GAPs are related to Ras GAPs. Yet both of these subclasses of proteins have been shown to function as GTPase activating proteins^{8,11–13}. In addition, other proteins, such as the products of JC99 and JC265, which have marginal similarity to GAPs also appear to participate in the biochemical pathway involving Ras¹⁴. Thus, based on the available sequences of GAP and GAP-like proteins, it seems that these proteins are quite divergent and that the assessment of their function solely on the basis of significant homology would be inadequate.

The model we have proposed to explain the lethal interaction between *pn* and *awd*^{K-pn} is not only based on sequence similarity but also draws upon our current understanding of these two loci. The lethality of *pn awd*^{K-pn} double mutants is apparently due to the expression of *Awd*^{K-pn} in combination with a lack of Pn (ref. 15). The *awd* locus encodes an NDP kinase³ and *awd*^{K-pn} is a missense mutation that appears to yield a protein product with a neomorphic function (A. Shearn, personal communication). It has been suggested that NDP kinases (like *Awd* and *Nm23*) regulate some critical biological processes, such as development and tumour metastasis, via a GTP-binding protein^{16,17}. This hypothesis is supported by studies that implicate NDP kinases in the effector activation of certain G proteins^{18–20}, and three different NDP kinases can activate the small G protein, ADP-ribosylation factor (Arf), by the direct phosphorylation of Arf-GDP to Arf-

GTP (ref. 21). Furthermore, contrary to Barnes and Bürglin's statement that *Awd* is principally microtubule-associated, immunological and biochemical studies indicate that only a small proportion of the enzyme appears to be associated with cytoskeleton while the most of it is found in other subcellular locations, such as the cytosol and nucleus (A. Shearn, personal communication). Because much of the non-microtubule-associated NDP kinase is probably also *Awd* protein (as homozygous *awd* mutants have less than 2% of the total enzyme activity of wild-type larvae³), this subcellular distribution suggests that *Awd* may provide NTPs for more than one biological process. In the light of this information, we have proposed a new model as one possible explanation of the *pn awd*^{K-pn} lethality. As with any model, its validity is subject to the rigours of testing by molecular, biochemical and genetic approaches.

Finally, Barnes and Bürglin state that our model fails to account for the pigment biosynthesis defect in the eyes of *pn* flies. But, as noted by Ruggieri and McCormick², it is conceivable that the hypothetical Ras-like G protein modulated by *Awd* and Pn is also involved in regulating the biosynthesis of pteridine pigments in *Drosophila*.

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Are *Anolis* lizards evolving?

SIR — Since Hurlbert¹ published his paper on experimental design in 1984, ecologists have been particularly careful to replicate their experiments. However, experiments without adequate replication are still occasionally performed, and the recent report of selection in *Anolis* lizards² is an example. This experiment involved measuring differential survival of lizards with respect to their morphology. Morphologically distinct lizards from four populations from different habitats (ecotypes) were maintained in separate enclosures in one habitat. There was significant differential survival of both males and females in just one of the enclosures. Because each ecotype was maintained in only one enclosure there was no replication within ecotypes. The key result was that selection (differential survival) occurred in the ecotype derived from the most distinct habitat.

The significant selection in the one enclosure could be due to this ecotypic difference, as the authors argue, but it may also be due to density (much lower

than in other enclosures), or average body size (much higher than in other enclosures). More seriously, it could be random. This can be illustrated using a thought experiment, such as the following 'fox-test': if one rogue fox that ate lizards and led to differential survival of different morphologies is dropped into the experiment at random, one-quarter of the time it will land in the enclosure that contains the ecologically most different morphotype, and all results follow. The probability of the association between intense selection and location is thus 0.25, which is clearly not significant. By this I do not mean to imply that foxes are the selective agent, but rather that something peculiar to one enclosure can cause the apparent selection. Replication of treatments over experimental units is the only way to estimate and control for such random variation. Inferential statistics such as ANOVA, which test for treatment effects, require an estimate of error within treatments. In this case, the treatment variable is ecotype, for which there is no replication (only one enclou-

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sure for each ecotype). The authors note that their study is a long-term experiment. Without alteration of the experimental design, future results may be suspect.

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SIR — Malhotra and Thorpe² provide no evidence for a rapid evolutionary response in natural lizard populations. Their experiment, which is conceptually ingenious and potentially important, was based on the translocation of four populations of *Anolis oculatus* from different ecosystems to four neighbouring arenas. The authors claim that there was rapid selective mortality in the translocated populations, and that this is evidence for the action of natural selection.

There are three problems with their experiment. (1) The design is what statisticians call confounded and ecologists pseudoreplicated. Because each population is in only one arena it is impossible to tell whether the claimed effect is due to differences between arenas or between populations.

(2) The correlation coefficient quoted in Fig. 2 is not statistically significant. There are not six degrees of freedom (as the authors appear to assume) but only two (there being four arenas). However, the figure does show a large response in one deviant population. As this montane population was from the ecologically most distinct site it could still support the authors' conclusion that the differential mortality is evidence of rapid evolutionary change due to natural selection.

(3) Unfortunately, even if we accept that the effect is due to the montane population responding differently to translocation, this does not necessarily imply natural selection. Using the values in Table 1 of ref. 2 we can calculate the average weight of the lizards placed in the different arenas: control 3.76 g, S. Caribbean 4.6 g, Atlantic 5.26 g, Montane 8.46 g. Lizards from the montane population were, on average, more than twice the weight of the controls when they were translocated. Because the morphological measurements taken have

not been corrected for body size or age, there is the strong possibility that it was the larger or older animals that died. No evidence is presented that the difference in body size between the populations is genetically based. We therefore reluctantly conclude that no reliable evidence of a rapid evolutionary response has been presented.

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THORPE AND MALHOTRA REPLY — Our study² shows clear evidence of rapid evolutionary response in lizards and Katti's and Lawton and McArdle's claims to the contrary are based on flawed logic and misunderstanding of the statistics used.

In particular, they reach their erroneous conclusions because they fail adequately to differentiate between the within-enclosure study of morphology, which supplied the critical evidence, and the between-enclosure study of 'fitness', which supplied only supplementary information. Their point that the difference between ecotypes could be due to chance differences between enclosures cannot pertain to the difference in the morphology of survivors and non-survivors within an enclosure, but only to the between enclosure analysis of 'fitness'.

Katti's claim that the "differential survival" of ecotypes could be due to density, average body size, or chance might have been true if we had measured differential survival as the relative number of survivors in each ecotype, but we did not do this. We measured the difference in morphology between survivors and non-survivors, and the fact that the most foreign ecotype exhibited a significant difference within an enclosure has to be due to differential selection, irrespective of any differences between enclosures, be they chance, average body size or density. Even so, biomass was constant between enclosures and density was lowest in the enclosure with greatest selection effects. Lawton and

McArdle's first point is rejected for the morphology on the same basis. The point regarding replication is valid for the fitness variables, but we are dealing here not with a small plot of ground in a suburban research station, but with about 100,000 m³ of remote natural forest per set of enclosures, requiring constant management. For several replications, the time required is prohibitive and the environmental perturbation caused is unacceptable, given the entirely supplementary and non-critical role of these fitness data. Moreover, a replicate in the Atlantic habitat supports our earlier results in showing a significant difference in morphology between survivors and non-survivors of foreign ecotypes and their lower fitness (growth rate, condition and weight change).

The average specimen size differs among ecotypes because size varies racially and not because of sample bias. There is no trend for preferential survival of a given size within an enclosure. There is no basis for excluding a single measure of size as it tends to be as highly heritable as other characteristics³⁻⁶. 'Size' has to be taken into account because of its intercorrelating effect, and this we do properly by using Mahalanobis D^2 (refs 7,8) as Lawton and McArdle should have realized. Consequently, the significant difference between montane-type survivors/non-survivors is unaltered by the exclusion of snout-vent length and/or size-adjusting the few linear measurements. This is the most important point because, as Lawton and McArdle admit, this significant difference is sufficient to prove our case.

With regard to the correlation between the dissimilarity in ecology and morphological dissimilarity between survivors/non-survivors, Lawton and McArdle do not appear to regard the D^2 for sexes as varying independently, even though it clearly does so. Random permutation techniques⁸⁻¹⁰ which give the probability of association when the data points are not independent, indicate a significant association ($P < 0.01$, 10,000 permutations). Moreover, if one accepts Lawton and McArdle's point, the morphological dissimilarity can be averaged across sex (giving four points) and the relationship linearized. This gives a correlation of $r = 1.0000$ which is significant whatever degrees of freedom are used.

Our morphological analysis provides sound evidence of rapid evolution in *Anolis oculatus* and our analysis of fitness, although non-critical, provides interesting information on the interface between evolution and ecology.

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Scientific Correspondence

SCIENTIFIC Correspondence is a relatively informal section of *Nature* in which matters of general scientific interest, not necessarily those arising from papers appearing in *Nature*, are published. Due to space limitations priority is usually given according to general interest and topicality, to contributions of fewer than 500 words, and to contributions using simple language. Contributions may be sent to referees and, in the case of matters arising from material published in *Nature*, are often sent to the author of that article for comment. □

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Mapping the world

Jean Michel Massing

The Heritage of Giotto's Geometry: Art and Science on the Eve of the Scientific Revolution. By Samuel Y. Edgerton Jr. Cornell University Press: 1991. Pp. 319. \$39.95.

WITH the Italian Renaissance, man became, in Leon Battista Alberti's words, "the mean and measure of all things". In the visual arts this led to a search for underlying principles of order and design, especially through the study of linear perspective and proportion. Edgerton's interdisciplinary study is a bold attempt to show how the perception of the world, as slowly refined by Renaissance artists, provided the impetus behind the scientific revolution.

Edgerton emphasizes the importance of euclidian geometry. Euclid's basic texts, the *Elements* and *Optics*, were translated from Arabic to Latin by the end of the twelfth century and were essential to treatises on perspective. For example, in *Unterweysung der Messung* (1525), Albrecht Dürer starts with the same axioms as does Euclid in *Elementa*. The new interest in mathematical principles led to a more realistic depiction of space, which Edgerton argues was fundamental to the rise of modern science.

He also stresses another Renaissance characteristic, the use of chiaroscuro (the artistic treatment of light and shade), as a prerequisite to later scientific development. Central to the Renaissance was also a new interest in naturalism. Around 1390–1400, the illuminators of scientific manuscripts began to test their artistic formulae against visual evidence, sketching plants and animals. This development probably reached its apogee a century later in Dürer's well-known watercolour *Large Piece of Turf* and his *Blue Roller* studies. Edgerton sees this new naturalism already exemplified by the star of the Magi in Giotto's *Epiphany* (1306), part of a fresco in the Scrovegni Chapel at Padua. Here he takes for granted the hypothesis previously proposed by Roberta Olson in *Scientific American* — that Giotto must have seen and drawn Halley's comet when it appeared in 1301 and used his drawings a few years later for the star of the Magi.

Certainly Giotto was the first artist to show the star of the Magi as a comet, but its colours — red, yellow and gold

against a blue background — hardly suggest a naturalistic rendering. More likely, as I have shown elsewhere, Giotto chose as his inspiration the comet *Miles* (Latin for 'soldier') that is com-



Dürer's woodcut *St John Devouring the Book* (1498) is a fine example of the trend among Renaissance artists to represent holy miracles as if they were explainable in mechanical terms.

monly described in mediaeval texts: it has the same shape and colours as Giotto's star of the Magi and is said to herald the end of great empires and the rise of new and greater powers. (For this reason, *Miles* was sometimes also connected with the death of Christ.) The first naturalistic drawings of comets made in Europe, in fact, were those by Paolo dal Pozzo Toscanelli who studied the path of Halley's comet when it reappeared in 1456.

The development of geometrical optics, which became known as *perspectiva* (from the Latin *perspicere*, 'to see through') is traced by Edgerton back to Robert Grosseteste, Bishop of Lincoln, and Roger Bacon. But the geometrization of pictorial space, he argues, pre-

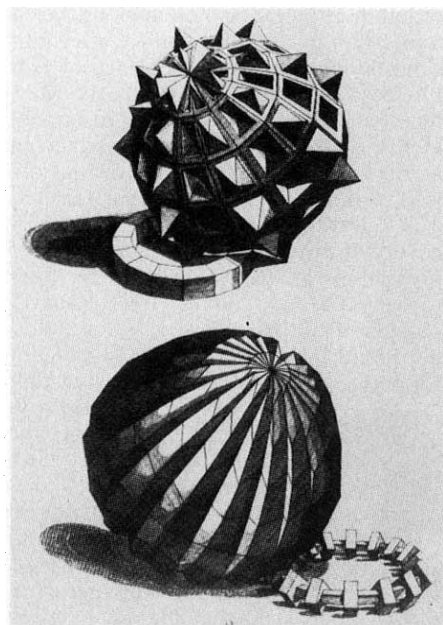
ceded their theoretical discussions, being found first in the thirteenth-century illusionistic cornices of the Basilica of San Francesco at Assisi in work by the Master of the Second Modillion (an anonymous artist, perhaps Giotto) and later in works indubitably by Giotto, such as the wall painting in the Scrovegni Chapel. The following century brought the discovery of linear perspective, a system of projection in which orthogonal lines focus at one point; this device, pioneered by Brunelleschi, is for example found in Masaccio's *Trinity* and Fra Lippo Lippi's *Annunciation*, a work that

Edgerton directly associates with Bacon's optical theory. But the relationship Edgerton proposes becomes rather far-fetched: does the descent of the Holy Spirit, represented by gilded dots entering the Virgin's body through a tiny opening in her garment, really symbolize the way in which visual rays enter the eye? It is surely far easier to see this image as a discreet symbol of the Virgin's womb.

Following the definition of a more realistic space in art came the geometrization of terrestrial space in cartography and technical drawings, such as those of Taccola, his follower Francesco di Giorgio Martini and, of course, Leonardo da Vinci. This new vision, together with the development of printmaking, greatly influenced the illustration of books on subjects such as ballistics, cartography, metallurgy and mathematics throughout the sixteenth century. Dürer, more than anyone, reflects this trend. His well-known treatises on measurement and human proportions were important for the scientific revolution; so was his approach, which discarded ideal proportion and ideal beauty for a strict anthropometry.

Edgerton also studies the geometrization of heavenly space in Raphael's *Disputa* fresco in the Stanza della Segnatura of the Vatican Palace, arguing that the fresco's setting is based on the semicircular frame of an armillary sphere with Christ at the centre. Here heaven and Earth are joined in a euclidian space.

But Edgerton's basic tenet is best illustrated by Galileo, whose interest in the arts is well known from his correspondence with the Florentine painter Cigoli. Galileo even applied for the position of *visitatore*, a kind of outside expert, at the famous Accademia del Disegno, the Academy of Art in Florence. His training, according to Edgerton, especially his interest in sciography, the 'art of shadows', led him to understand



An engraving from Lorenzo Sirigatti's *Pratica di prospettiva* (1596) used to illustrate chiaroscuro.

the nature of the Moon, which he recorded in his wash drawings based on observations made with a telescope in November and December 1609. Galileo's conclusions are famous: "I have been led to the . . . conviction that the surface of the moon is not smooth, uniform and precisely spherical . . . but it is uneven, rough, and full of cavities and prominences, being not unlike the face of the Earth, relieved by chains of mountains and deep valleys".

Finally, Edgerton bolsters his basic hypothesis by using it to show why Chinese scientists, the inventors of gunpowder, printing and the magnetic compass, did not attain the logico-mathematical conditions of a scientific revolution in the seventeenth century. This provides a fitting conclusion to an ambitious and largely persuasive book. □

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Particles to the rescue

Michael L. Cherry

Neutrino Physics. By Klaus Winter. Cambridge University Press: 1992. Pp. 670. £75, \$125.

It is appropriate that Klaus Winter has published this authoritative and comprehensive book on neutrinos within a few months of the announcement that this year's prestigious Panofsky and Sakurai prizes have been awarded to Ray Davis, Fred Reines and Lincoln Wolfenstein for their contributions to the physics and astrophysics of neutrinos. The idea of the neutrino was invented by Wolfgang Pauli in 1930 as "a desperate remedy" to save the law of conservation of energy in nuclear β -decay. But it took a quarter of a century before Reines and Cowan finally presented evidence for the direct detection of the neutrinos (actually the electron antineutrinos) produced by the Hanford and Savannah River reactors. And it was only in 1962 that Lederman, Schwartz, Steinberger and their colleagues used a high-energy accelerator beam at Brookhaven to demonstrate the difference between electron and muon neutrinos. Since then, experiments at reactors, accelerators, underground and on the Earth's surface have been done to study the neutrino's properties and to use the neutrino as a probe of weak interactions and subnuclear structure.

Recently, experiments at CERN and Stanford have led to the important discovery that there are three (and only three) families of neutrinos. Unlike any other particle, the neutrino is subject to

only the weak nuclear interaction and is therefore a unique probe of the weak nuclear force. Nevertheless, the structure of the three neutrino families closely parallels the structure of the three families of quarks. But the quark families are not distinct (they mix together), so the natural suggestion is that the neutrinos mix as well (despite the experimental evidence so far to the contrary). For 50 years, though, studies of the weak interaction have proceeded on the assumption that neutrinos are neutral, highly penetrating and massless. Mixing, on the other hand, requires neutrinos to have some mass.

The neutrino mass is clearly small. Direct studies of the endpoint energy in tritium β -decay set an upper limit to the electron antineutrino mass of less than 10 electronvolts, a factor of 50,000 less than the mass of the corresponding charged electron. This is consistent with the arguments from cosmology, the observations of neutrinos from the 1987 supernova in the Large Magellanic Cloud, and the inability so far to find neutrinoless double β -decays. Solar neutrino experiments, however, seem to be suggesting the existence of mixing at mass levels near 10^{-4} electronvolts.

If the neutrino is massive, and in particular if it is a Majorana particle (that is, if the neutrino is its own anti-

particle), then the origin of the neutrino mass may be related to the existence of new physics at energies beyond the 10^{12} -electronvolt energy scale to which accelerators have so far allowed us to probe. In other words, the neutrino mass is small because there is some new mass scale (presumably related to grand unification) that is large. Much of the current interest in neutrinos stems from the hope that laboratory and astrophysics studies of relatively low-energy neutrinos may actually shed light on fundamental new physics beyond our standard model of particle physics and at energy scales beyond those of current or planned accelerators.

Professor Winter is a leader in accelerator studies of neutrinos and weak interactions. As a senior member of the CERN Laboratory in Geneva, he has assembled an expert, generally well-written and exceedingly well-planned set of essays into a very useful overview of neutrino physics and astrophysics. The profusion of excellent recent books on neutrinos is a reflection of the topic's popularity. Winter's book, however, fits into its own special niche. With an emphasis on the experimental results and their significance, *Neutrino Physics* begins with a historical review by Pauli himself (translated here for the first time into English) and steps through the individual experiments up to the observation of neutral currents in CERN's Gargamelle detector. This introduction is followed by a combination of chapters on the theory and experiments relating to the fundamental properties of neutrinos, including discussions of the differences between Majorana and Dirac neutrinos, double- β -decay and tritium-endpoint experiments, oscillations and neutrino counting, and astrophysical and cosmological constraints.

Even at 600 pages, the book cannot cover everything: nuclear β -decay and standard Fermi theory, for example, are deliberately omitted because fine treatments of these topics can be found elsewhere. But there is an extensive discussion of neutrino interactions in matter followed by a series of articles on accelerator studies of the weak interaction and nucleon structure, and a concluding section on solar neutrinos, supernovae and cosmology. A second volume, on neutrino detectors and neutrino beams, is promised. If it is anywhere near as thorough, complete and as useful as this first volume, I look forward to seeing it too. □

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■ New from World Scientific is *The Relations of Particles* by L.B. Okun, a collection of reviews on elementary particle physics £22.

Lessons from a grand master

John C. Gerhart

The Triumph of the Embryo. By Lewis Wolpert. Oxford University Press: 1991. Pp. 244. £14.95, \$22.95.

LEWIS Wolpert is well known to developmental biologists for his experiments on sea-urchin gastrulation, hydra regeneration and limb development. He is also renowned for his inspiring generalizations about 'positional information' in the late 1960s, in particular his statement that an embryonic cell's genome and developmental history provide the choices, but its position is the chooser. His work and writing stimulated a generation of developmental biologists to turn to experiments on pattern formation. It is noteworthy that he has now written a book "which enables nonspecialists to learn how embryos develop". Such books are rare, perhaps because an understanding of developmental biology not only requires knowledge of the differently changing embryos of many different groups of organisms, but also nowadays draws on the fundamentals of many other areas of biology, such as genetics, molecular biology and cell biology. To make the subject comprehensible, Wolpert repeatedly stresses his belief that unifying principles underlie the differences and complexities. Of course, it takes someone of Wolpert's purview to discern these principles.

Throughout, he remains true to his objective of explaining how embryos develop. He makes little attempt to hold one's attention with stories of scientists and discovery, or with the history of ideas in the field, or with anecdotes about strange creatures. Nor does he resort to simple analogies to explain difficult ideas. There is no speaking down to his nonspecialist reader. In the absence of these devices, the book reads like a serious text. The ideal nonspecialist reader might be a bright secondary-school pupil or first-year university student, or someone with a

training in another aspect of biology, for example medicine, who wants to become acquainted with the facts and ideas of developmental biology. Specialists may also enjoy the book (if merely to see how well Wolpert renders their subject).

The range of topics is enormous: cleavage of the egg into cells, morphogenesis, pattern formation (positional information and induction), limb development, the role of DNA in development (the developmental programme), cell and tissue differentiation, *Drosophila* development (currently the



A mouse embryo at 15 days of gestation (reproduced from *Molecular Biology of the Cell* by Bruce Alberts et al., published by Garland).

best understood case), brain development, sex differentiation, growth, cancer, ageing, regeneration and evolution. Experimentation, description and interpretation are well balanced in the presentation which is organized around Wolpert's generalizations about development, in particular around the concepts of positional information and developmental programmes.

I have a few criticisms of this organization. First, although positional information works well as a unifying concept, the idea of developmental programmes comes across less well. The idea of a 'programme' as a series of operations is introduced early on but it becomes increasingly hard to guess the meanings of the many shadings of the term throughout the book: developmental programmes, generative programmes, the cell's

programme, genetic programmes and internal programmes. (In the last chapter, Wolpert reveals that "[t]he generative program is essentially contained within the genes", a statement about which the nonspecialist reader may want much more said, much earlier on.) Second, oogenesis is largely omitted as a topic, leaving the reader unenlightened as to where eggs come from and how the embryo establishes its first regional differences. Finally, the notion of genomic equivalence might have been presented earlier in the book (it is not really made explicit until about a third of the way through) to prepare the reader for the problem of the selective and position-dependent readout of genetic information in development.

This is a clear and engagingly written book, recommended certainly to nonspecialists, but also to developmental biologists who wish to see how a senior figure in the field can bring order to a chaotic subject without forcing his opinions on the reader. Above all, Wolpert loves his subject and cannot help but share his enthusiasm with us. □

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■ Oxford University Press have also recently published *Transdifferentiation: Flexibility in Cell Differentiation* by T. S. Okada. Most of the studies that Okada mentions are at the cellular level. £45.

Hyperinteractive

Robert Temple

The Hypnotic Brain: Hypnotherapy and Social Communication. By Peter Brown. Yale University Press: 1991. Pp. 322. £25, \$40.

THIS is a book ruined by a preconception. The author is determined that hypnosis shall be a "socially-interactive phenomenon". The baleful influence of the late Milton Erickson, a brilliant clinician but shaky theorist, is at work here. And there is too much reliance on the work of Nicholas Spanos, which in my opinion is unconvincing and tendentious. It might be said that Brown is not attentive enough to other schools of thought. Why does he pay so little attention to Ernest Hilgard, a giant in the field?

Brown makes much of the view that multiple-personality disorder is also 'socially-interactive'. He says that "[s]ocial roles must be constantly renegotiated" and speaks of the need for alternative personalities to "rehearse themselves" and evolve as social products. An alternative personality needs "an initiation process and progressive rehearsal of the role". In my view, this is not only nonsense but potentially dangerous non-

sense. As someone who had a parent with multiple-personality disorder, I can absolutely assure Brown that initiation and progressive rehearsals are not necessary and that to view the disorder as requiring them because of a preconception could lead to wrongly conceived treatment and possibly disastrous results.

Brown gives a superficial impression of having studied the history of multiple-personality disorder, but in fact all his knowledge of the early period seems to be derived from a single book by William James. No works by Morton Prince, Pierre Janet, Boris Sidis or Alfred Binet are listed in his bibliography. Indeed, there is nothing listed by John Watkins, perhaps the United States' leading expert on this subject at the moment.

A strong protest must also be made against the use by Brown of a sloppy technique known as 'token referencing'. The book is peppered with contentious statements followed by names and dates placed in parentheses. Very rarely does Brown give a page number. This technique has spread like a cancer through academic and historical literature, and it is sad to see its spores germinating in books purporting to be scientific. The result of token referencing is that the labour of checking references far exceeds the labour of reading the book, and can become impossible. (With historians dealing with manuscripts this is often by design.)

It is a great pity that so much fascinating material as is to be found in this book is shaped around the theme that "we can consider hypnosis not as a single entity but as one culturally defined context of a rhythmic biologic opportunity to enter into a number of different states of consciousness. There do not appear to be any reliable psychological or physiologic concomitants of trance that are unique to hypnosis. . . ." For it has been known for years that electroencephalogram readings show trance to be a state distinct from sleep, waking or meditation. In one of the most interesting sections of the book, Brown surveys work on ultradian rhythms of the brain, but his survey of hemispheric lateralization is not up to date and as a result is misleading.

It is marvellous that he includes in a book on hypnosis discussion of tribal trance material from anthropologists, and a great deal of discussion of neurophysiological matters. These are extremely important and all too often lacking from hypnosis books. Neurotransmitters, subcortical functions, nasal airflow, muscle paralysis in sleep and the "basic rest activity cycle" are also welcome items for consideration in the book. But they are not digested properly by Brown, and he gives the impression of having rushed into print too soon.

And his conviction that "though there are changes in brain function in hypnosis, they are not unique to hypnosis" gets us ultimately nowhere. He believes hypnosis is simply "a complex cognitive task". So is trying to make sense of Brown's position. □

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Kindred spirits

Linda Partridge

Kin Recognition. Edited by Peter G. Hepper. Cambridge University Press: 1991. Pp. 457. £60, \$89.50.

ANIMALS are capable of prodigious feats and dismal failures in recognizing their kin. A Mexican free-tailed bat mother can find her own offspring in a cave containing a million bats, whereas a hedge-sparrow mother fails to recognize a cuckoo egg in her nest, even though it is coloured quite differently from her own eggs. This volume provides 14 accounts of mechanisms and consequences of kin recognition in animals, some dealing with particular taxa such as amphibians and primates, others with particular issues such as fetal learning.

Two of the key issues in kin recognition covered in the book are whether prior familiarity with relatives is necessary and, if so, how this information is used. In contrast to Oedipus, sweat bees and many other Hymenoptera can discriminate on the basis of smell between related and unrelated individuals without any prior experience of conspecific individuals. Young adults nonetheless learn the odour of the colony and of the brood. How these sources of information are used under natural conditions is not known; several of the book's contributors point out that information that can be shown to be present in one context may fail to be used in others, or may be acquired or used only if the normal source of information is absent. Disentangling these issues in the complex environment of chemical communication in social insects is not easy. M. H. Johnson provides an excellent account of similar issues in filial imprinting in birds, a clear example of predispositions to learn to recognize particular stimuli. A general conclusion is that animals can gather unused information about kin, which may be selected for use in other contexts.

As in the oedipal myth, failure to recognize kin can have two consequences: incest and neglect of relatives.

Incest can be harmful because it causes 'inbreeding depression', that is, it decreases the progeny's fertility or likelihood of survival. C. J. Barnard and P. Aldhous provide a useful review of how nonrandom mating arises from selection to avoid inbreeding, and of the difficulties of distinguishing this cause from others such as dispersal, population structure and sexual selection. For example, much work shows that mice in the laboratory can discriminate on the basis of smell between mice carrying different major-histocompatibility-complex alleles and that females can use this complex as a cue to block implantation of a litter sired by the previous mate if they are exposed to a strange male. But it is not clear whether this system is used to avoid inbreeding or, indeed, whether it is used at all in the wild.

M. D. Beecher elegantly analyses the conditions under which bird parents might be expected to recognize their offspring. Interspecific cross-fostering experiments with avian chicks are almost always successful, showing that bird parents do not discriminate between their own young and those of others, the exceptions occurring for species where the young birds naturally move between nests. For most bird parents, young in the nest are overwhelmingly likely to be offspring, and the costs of rejecting an offspring are greater than those of accepting an unrelated individual. Failure to reject strange young need not imply an inability to recognize offspring. Rough-winged swallows will feed bank swallow chicks, but double-take before doing so, and will feed their own young in preference if given a choice between them and young bank swallows. Young birds before fledging may often benefit from not being recognizable to adults — presence in the nest is enough of a cue for parental purposes, and if a chick does accidentally wander it will be less likely to be attacked and more likely to be adopted by non-parents. Young birds often become recognizable to their parents after fledging because their location is no longer a reliable cue to their identity.

The book is useful, well edited and carefully balanced. If there are omissions, they are the absence of clear chapter summaries and the lack of any discussion of plants or unicellular organisms. The mechanisms of self-incompatibility in plants are now being unravelled at the molecular level, and unicells provide many fascinating examples of the rules of chemical warfare between different clones. □

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Genetics and speciation

Jerry A. Coyne

Called the "mystery of mysteries" by Darwin, speciation is still a little-understood area of evolution. Genetic analysis, however, has yielded new generalizations about speciation and suggests promising avenues of research.

ONE of the great problems of biology is how a continuous process of evolution can produce the morphologically discontinuous groups known as species. Despite its title, *On the Origin of Species* made few inroads on this problem, which remained until Dobzhansky¹, Muller² and Mayr³ proposed the genetical theory of speciation that is still widely accepted. Considerable dispute remains, however, about the details of this theory. How many genes must change to make a new species? Do these genes occur in consistent positions on chromosomes? Are conventional changes in allele frequency all that is involved, or are new evolutionary processes called for? What are the relative roles of natural selection and genetic drift? How important is geographical isolation?

Because speciation usually occurs slowly, it is hard to resolve these questions through direct observation, but the process can be studied through the patterns it leaves behind. Here I discuss the genetic patterns involved, concentrating on the numbers, locations, and effects of genes that produce reproductive barriers between species. Although these studies began in 1936 (ref. 4), their proliferation in the past 10 years has helped resurrect speciation as a major issue in evolutionary biology. This work has yielded intriguing generalizations about speciation and has enabled us to winnow the tractable from the intractable questions. Moreover, the advent of molecular methods for counting, mapping, and identifying the genes involved in speciation offers a useful new approach to the problem.

Much work in this area is deeply embedded in historical controversy, so I shall briefly review species concepts and theories of speciation before turning to the genetic data.

The neodarwinian view

Most evolutionists adhere to Mayr's 'biological species concept' (BSC)³: species are groups of interbreeding populations that are reproductively isolated from other such groups. The reproductive barriers separating members of different species are divided into 'prezygotic isolating factors' (mate discrimination, different habitat preferences, pollination by different insects, and so on), and 'postzygotic isolating factors' (hybrid inviability and sterility). It is this reproductive isolation, in concert with selection and genetic drift, that creates and expands the morphological gaps between species living in the same area⁵. Although new species concepts arise with alarming regularity, the BSC has one advantage over its competitors in that it suggests a straightforward research programme: when we understand the origin of reproductive isolating factors, we understand the origin of species.

The neodarwinian view of how these factors arise requires geographic barriers between conspecific populations. This physical isolation ('allopatry') leads inevitably to evolutionary divergence through natural selection or drift. After sufficient time, reproductive isolation evolves as a byproduct of this genetic differentiation. Hybrid inviability, for example, may arise from diverged developmental systems that do not cooperate in a single genome.

Although reproductive isolation may be perfected during the period of geographical isolation, it is often suggested that natural selection on incompletely isolated populations that become 'sympatric' (occupy the same area) could complete speciation

by fixing alleles that reduce interspecific mating and hence minimize the number of unfit offspring. This process, called 'reinforcement', was once thought to be an important phase of speciation, but its ubiquity is now questioned because theoretical models show that reinforcement is unlikely^{6,7}. Moreover, there are only a handful of studies showing that, in species with overlapping ranges, individuals from the area of overlap have the highest levels of interspecific mate discrimination in the laboratory⁷⁻⁹. One recent survey of *Drosophila*, however, showed that sexual isolation was much higher between sympatric than between allopatric species pairs of similar age¹⁰ (Fig. 1). Many more such studies are needed to determine the importance of reinforcement.

The extensive biogeographical evidence for allopatric speciation is summarized by Mayr^{3,11}. There are persistent claims, however, that geographic isolation is not in fact necessary, and that 'sympatric speciation' may occur within a single interbreeding population. The likelihood of this process remains one of the most contested aspects of speciation¹²⁻¹⁴. Undoubtedly genetic divergence occurs much more easily when populations are disjunct, but some models and experiments show that populations can develop reproductive isolation while remaining in potential contact¹⁵⁻¹⁸.

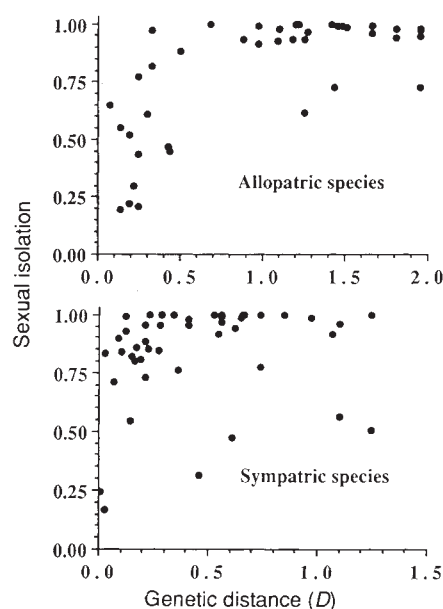


FIG. 1 Sexual isolation between allopatric (top) and sympatric (bottom) pairs of *Drosophila* species. Each pair is represented by one point, indicating both Nei's electrophoretic genetic distance between the species⁷⁸ and their degree of sexual isolation as measured in laboratory mating experiments. Genetic distance measures the divergence of allozyme frequencies, being 0 when species are identical at all loci. Sexual isolation ranges from 0 (species mate randomly with each other) to 1 (no interspecific matings). For closely related species ($D < 0.5$), sympatric species have a significantly higher level of sexual isolation than allopatric species, possibly indicating that natural selection has reinforced sexual isolation in sympatry. See ref. 10 for fuller discussion of the data and results.

There is one recent demonstration that the initial steps of sympatric speciation, niche polymorphism with reduced gene flow, can occur in nature: the occurrence of genetic differentiation between sympatric 'host races' of the North American tephritid fly *Rhagoletis pomonella*¹⁹. For most sympatric species, however, nothing is known of the history of speciation, and it is always possible to construct a story involving geographic isolation in the unknown past. It is thus impossible to estimate the importance of sympatric speciation in nature.

Surprisingly, there is almost no empirical evidence for the most crucial aspect of allopatric speciation, the origin of reproductive isolation as a byproduct of drift or selection. The widespread acceptance of this idea comes from observing pre- and postzygotic isolation in crosses between allopatric populations, and from the idea that genetic divergence must eventually produce sexual isolation or developmental problems in hybrids. There are two laboratory studies showing reproductive isolation as a byproduct of adaptation^{20,21}, but only one example from nature: genes conferring copper tolerance on a mine-dwelling population of the monkey flower, *Mimulus guttatus*, are lethal in interpopulation hybrids²².

Models

The geography of speciation tells us nothing about its genetic basis, but it is clear that reproductive isolation must usually be based on more than one gene. A simple two-locus model of reproductive isolation, proposed by Dobzhansky¹ and Muller², involves an ancestral species of genotype $A_1A_1B_1B_1$ that evolves to $A_1A_1B_2B_2$ in one population and $A_2A_2B_1B_1$ in another. Speciation would result if epistatic interaction between the A_2 and B_2 alleles caused complete sterility or inviability of hybrids. In such cases hybrid inferiority evolves between species without ever appearing within species: neither population need cross an 'adaptive valley'. Similar models are possible for sexual isolation, involving the interaction between genes affecting male characters and those affecting female preference.

There is no doubt that such epistatic interactions exist: complementary lethals like alleles A_2 and B_2 have been described in plants and *Drosophila*^{2,22}. Reproductive barriers could, however, be caused by many more than two genes. Ethological, temporal, or habitat isolation, for example, may result directly from selection on ecological traits. The neodarwinian view that adaptation is usually polygenic would then predict that these isolating factors would also be polygenic. Unfortunately, genetic models of speciation have not progressed much beyond the simple two-locus scenario. More elaborate models require assumptions about the numbers, effects and interactions among genes causing reproductive isolation, information that exists for only a few species.

Data

Genes contributing to reproductive isolation may continue to become fixed after speciation is complete, and it is not easy to identify those involved in speciation itself. This problem might be addressed by correlating the amount and nature of reproductive isolation between species with an estimate of their divergence time from molecular data¹⁰, or by analysing allopatric populations showing only one form of reproductive isolation²³. Mate discrimination may be a primary isolating factor in animals: cases of rampant speciation, such as Hawaiian *Drosophila* and New Guinea birds of paradise, often involve groups that have extreme sexual dimorphism or elaborate mating rituals. This may, however, only reflect the ease of detecting species in such groups.

How many genes cause speciation? Although changes in at least two genes are usually required for speciation, it is important to know whether many more loci are involved. As noted above, the neodarwinian view predicts that many isolating factors would be polygenic. Complete sexual or temporal isolation based on only two or three genes might then imply rapid speci-

ation, perhaps through strong natural selection. The pattern of gene interaction, moreover, might allow one to reconstruct the sequence of genetic changes causing isolation²⁴.

These questions are best answered by brute-force genetics: crossing closely related pairs of species whose reproductive isolation can be overcome in the laboratory, and observing the segregation of genes causing isolation in the backcross or F_2 hybrids. The number and location of 'speciation genes' is then estimated by observing their association with mapped morphological or biochemical mutations. Such studies must be limited to genetically well characterized species like mice or *Drosophila*. The advent of DNA markers²⁵, however, considerably expands the number of species available for analysis and the number of genomic segments that can be examined, although no such surveys have yet been done.

The few experimental analyses give mixed results. In *Drosophila*, the most-studied group, reproductive isolation is often polygenic. When one isolating factor is fairly strong, several to many genes are usually involved²⁶⁻³⁴. Most of these studies revealed the largest number of genes that could be detected with the chromosome markers used, with lower bounds between five and nine loci.

Some cases, however, involve fewer genes. In the most extreme example known, prezygotic isolation in snails is apparently caused by single mutations that change the direction of coiling, making copulation impossible. Fixation of these genes depends critically on the maternal inheritance of the alleles and limited mobility of the snails³⁵. Occasionally, only a few segments of the genome cause reproductive isolation, implying that a few loci are involved³⁶. Among twelve large chromosome segments examined in *D. pseudoobscura*, for example, only four cause male sterility in hybrids with its subspecies *bogotana* (H. A. Orr, personal communication). There are three other cases in which a given isolating factor is probably due to three or fewer loci: hybrid inviability caused by tumour formation in swordtail/platyfish hybrids³⁷ (two genes), ethological isolation caused by sex pheromone production and perception in the corn borer²⁴ (three genes), and inviability in population hybrids of *Mimulus guttatus*²² (two genes).

Several points must be considered when interpreting these results. When an isolating factor is polygenic, direct genetic analysis is not powerful enough to distinguish between 9 and 900 genes because of the limited number of genetic markers. A statistical approach combining genetic and biogeographic information from hybrid zones can produce much larger estimates: at least 150 genes causing inviability in hybrids between two chromosomal races of the grasshopper *Podisma pedestris*³⁸, and at least 55 genes reducing the fitness of hybrids between the toads *Bombina bombina* and *B. variegata*³⁹. These figures are remarkable, given that hybridization still takes place in nature and that (according to molecular evidence) the races of *Podisma* arose quite recently.

The genetics of a single isolating factor is not equivalent to the genetics of speciation, which may involve many factors. Inviability in swordtail/platyfish hybrids, for example, may have been unimportant in their speciation, because sexual and habitat isolation usually prevent hybridization in nature (K. Kallman, personal communication). Moreover, for species that diverged long ago, genetic analysis may overestimate the number of genes involved in speciation because most of the change may have followed the speciation event. So, for example, the estimate of nine or more loci causing hybrid inviability between the long-diverged species *D. melanogaster* and *D. simulans*⁴⁰ may exceed the number producing the initial inviability.

Until such genetic studies are extended to groups other than *Drosophila*, and to other isolating factors (we have no information, for example, on the genetics of temporal or habitat isolation), we cannot generalize about the number of genes causing speciation. We can conclude, however, that reproductive isolation sometimes has a relatively simple genetic basis.

Mapping 'speciation genes': X-chromosome effects and Haldane's rule. The chromosomal mapping of 'speciation genes' is in its infancy, but has already produced one regularity: the sex chromosomes have a disproportionately large effect on hybrid sterility and inviability. This result holds in every one of 24 genetic analyses, including species as diverse as tsetse flies, mice, butterflies and *Drosophila*^{34,41,42} (see Fig. 2, for example). Most of these cases involve sterility of the heterogametic sex, but the pattern also holds in homogametic hybrids. This large effect of the X chromosome is not regularly seen for prezygotic isolation or morphological differences among species⁴³.

It is rare to find an evolutionary pattern with so few exceptions. Moreover, this pattern probably explains another well-known observation called Haldane's rule⁴⁴: when only one sex is sterile or inviable in the offspring of species crosses, it is nearly always the heterogametic sex. This rule also has very few exceptions (Table 1). Because it is obeyed regardless of which sex is heterogametic (males in flies and mammals, females in birds and butterflies), Haldane's rule must be based on the sex chromosomes themselves and not on the sensitivity of one gender to hybridization.

As heterogametic sterility and inviability is the first step in the evolution of complete sterility and inviability¹⁰, an explanation of Haldane's rule and sex-chromosome effects would clarify one of the earliest stages of speciation. Genetic analysis has ruled out two obvious explanations: heterogametic but not homogametic hybrids are sterile because only the former have a genetic imbalance between X chromosomes and autosomes⁴⁵, and that the X chromosome simply carries more genes that can mutate to sterility or inviability within a species⁴¹. Several other suggested explanations also seem unlikely⁴¹.

The remaining viable explanations fall into two categories. The first proposes that X-chromosome effects are due to special developmental properties of that chromosome, such as its involvement in dosage compensation, its inactivation in mammalian spermatogenesis⁴⁶, or its interaction with the Y chromosome⁴⁵. The second group proposes that because the X chromosome is hemizygous and lacks recombination in one sex, it accumulates some evolutionary changes faster than do the autosomes, and that these changes may cause sterility or inviability of hybrids. Evolution of this type could be based on the substitu-

tion of underdominant or advantageous partially recessive alleles^{41,43}, or of meiotic-drive alleles that cause one sex chromosome to eliminate the other^{47,48}.

Unfortunately, none of these theories is completely satisfactory. Some fail to account for the facts. Theories based on the susceptibility of spermatogenesis to genetic divergence do not explain why it is the female hybrids that are sterile or inviable in birds and Lepidoptera, nor why the sex chromosomes have a large effect on postzygotic isolation in homogametic females. Theories based on deleterious interactions between X and Y chromosomes do not explain why the Y chromosome often has no effect on hybrid inviability or sterility^{41,49}, nor why XO species obey Haldane's rule⁴¹. Theories attributing postzygotic isolation to X-Y meiotic drive cannot explain the large role of the X chromosome on postzygotic isolation in the *homogametic* sex and on inviability of either sex (meiotic drive does not cause inviability), the absence of meiotic drive in semisterile hybrid males⁵⁰, and the frequent lack of a Y-chromosome effect on postzygotic isolation. Other theories require unproven assumptions, such as the recessivity or underdominance of genes causing sterility or inviability⁴¹.

It is also possible that Haldane's rule does not have a single explanation, but is a coincidental result of diverse phenomena. Hybrid inviability and sterility may have different causes, as may disruption of spermatogenesis in flies and oogenesis in butterflies. Sorting out these possibilities must be a major goal of evolutionary genetics.

Molecular analysis of speciation genes. It is hard to map genes affecting characters like habitat preference and mating behaviour, and genes that are easier to map, such as those causing hybrid sterility or inviability, are difficult to isolate because their phenotype appears only in hybrids. Nevertheless, understanding such isolation could resolve several questions about speciation. Is reproductive isolation caused by conventional mutations in genes, or by novel genetic elements such as repeated sequences or transposons^{48,51}? When real genes are involved, have they diverged in coding sequence or in noncoding regulatory regions⁵¹? Do these genes have any normal function within species? Is hybrid male sterility, for example, due to genes involved in spermatogenesis, or to genes expressed in both sexes that disrupt development only in males? If both

FIG. 2 Sterility of backcross males in *D. pseudoobscura*/*D. persimilis* hybridization, showing large effect of the X chromosome³¹. F₁ hybrid females are backcrossed to *D. pseudoobscura* males, and genotypes of backcross males are identified by morphological mutations (chromosomes of *D. pseudoobscura* are shown in white, of *D. persimilis* in black). The fertility of a genotype is measured as the proportion of individuals having at least one motile sperm. The difference in sperm motility between the first eight and the second eight genotypes shows the nearly complete effect of the X chromosome.



males and females are sterile or inviable, are the same genes responsible in both sexes? (There is preliminary evidence from *Drosophila* against this view^{52,53}.) If only a few genes are involved, does their function give a clue to the order in which they diverged?

Only two speciation genes have been isolated, one of which gives an unexpected result. Genes producing pigment spots in the platyfish cause fatal melanomas in hybrids with swordtails. This dysfunction involves an X-linked oncogene, producing a receptor tyrosine kinase, that is normally suppressed in the platyfish by a dominant autosomal allele. The swordtail, however, lacks the X-linked tyrosine kinase locus and the dominant suppressor³⁷. Backcross or F₂ hybrids may inherit the oncogene without the suppressor, producing severe malignancy. This form of isolation is therefore caused not by a change in gene sequence, but by the complete absence of a locus in a related species. The other speciation gene, the X-linked *per* locus in *Drosophila*, has apparently changed by more conventional means. The *per* locus affects the rhythm of wing vibration during male courtship, a character influencing the female's willingness to mate⁵⁴. The difference in rhythm between *D. melanogaster* and *D. simulans* is caused by one to four base substitutions in the coding region of the gene⁵⁵.

Three other loci, all in *Drosophila*, have been mapped closely enough to make them candidates for molecular analysis. These include an X-linked gene that causes male sterility in *D. simulans*/*D. mauritiana* hybrids⁵⁶, and a gene on the tiny fourth chromosome of *D. simulans* that causes male sterility when introgressed into *D. melanogaster*⁵⁷. Finally, there is the remarkable *hybrid male rescue* (*Hmr*) gene⁵⁸, whose mutation rescues sexes that are normally inviable in crosses between *D. melanogaster* and its relatives. This may not, however, be a gene whose evolution produced reproductive isolation, but simply a switch that overrides the many loci known to cause inviability in these hybrids⁴⁰.

Do unusual genetic phenomena cause speciation?

Reproductive isolation may sometimes be based on inherited elements that are not conventional genes. Chromosome doubling in hybrids (allopolyploidy) is a major cause of speciation in plants⁵⁹. It has been suggested that rearrangements of single chromosomes are equally important in animal speciation, because some heterozygous rearrangements make their carriers semisterile⁶⁰. This suggestion, however, has many problems¹². Two more recent ideas bear special attention. It has been widely proposed that postzygotic isolation could be caused by the uncontrolled movement in hybrids of transposable genetic elements that are normally immobile within a species^{48,51}. This model is based on the phenomenon of hybrid dysgenesis in *D. melanogaster*, in which various anomalies, including sterility, appear in hybrids between strains containing transposons and those lacking them. Hybrid sterility and inviability factors, however, map to consistent chromosomal sites (particularly on the X), which is not expected if mobile elements are involved. Moreover, species hybrids do not show other phenomena associated with transposon movement, such as elevated mutation rates and male recombination in *Drosophila*^{30,61,62}.

A form of isolation that may be more widespread is hybrid inviability caused by infectious bacteria. Described in five orders of insects⁶³, this phenomenon was discovered when normally inviable hybrids were rescued by rearing parents on food containing antibiotics. (All of these cases involve the bacterial genus *Wolbachia*.) Infection of a single species produces inviability in only one direction of a cross, but different infections in geographically isolated populations can cause partial or complete reproductive isolation, which has occurred in *Drosophila simulans*⁶⁴, the wasp *Nasonia vitripennis*⁶⁵, and the mosquito *Culex pipiens*⁶⁶. The striking aspect of infectious speciation, which makes it similar to polyploid speciation in plants, is that it is virtually instantaneous and occurs without genetic change

TABLE 1 Conformity of species hybridizations to Haldane's rule

Group	Trait	Hybridizations with asymmetry	Number obeying Haldane's Rule
<i>Drosophila</i>	Sterility	114	112
	Inviability	17	13
Lepidoptera	Sterility	11	11
	Inviability	34	29
Birds	Sterility	23	21
	Inviability	30	30
Mammals	Sterility	25	25
	Inviability	1	1

'Hybridizations with asymmetry' refers to those interspecific crosses in which hybrids of one sex are less fertile or viable than hybrids of the other sex. Females are heterogametic in birds and lepidoptera, homogametic in *Drosophila* and mammals. Other groups, not shown above, also follow Haldane's rule. See ref. 41 for sources of these data.

in the host. Although endosymbionts have been proposed as a universal cause of speciation⁶⁷, this is disproven by many studies showing that reproductive isolation maps to chromosomes. Nevertheless, cytoplasmic effects on postzygotic isolation are not rare⁴⁹, and antibiotic treatment should be part of any genetic analysis.

Natural selection or genetic drift?

Although it is difficult to reconstruct the sequence of genetic changes leading to speciation, it is even harder to discover what causes those changes. It seems obvious that natural or sexual selection could yield reproductive isolation as a byproduct, but some have suggested that speciation may often depend on an interaction between selection and genetic drift^{11,68,69}.

These theories of speciation were proposed to explain two observations. First, isolated species, such as those on oceanic islands, are often morphologically more extreme than related species on continents (Hawaiian *Drosophila* and Pacific island birds are the classic examples). Second, speciation in such isolates may be very rapid. Because such species may originate after colonization by a few founders, they could experience large changes of allele frequency from random genetic drift^{11,70}. This could cause substantial morphological change and reproductive isolation, propelling populations across adaptive valleys that could not be crossed by selection alone. The joint role of drift and selection distinguishes these 'founder-effect' theories from more traditional views of speciation caused by drift or selection acting independently. Both old and new theories can, however, account for the intriguing observation that laboratory populations repeatedly reduced to a few individuals may develop weak reproductive isolation^{71,72}.

There is no doubt that speciation on islands often begins with a few colonists that experience genetic drift, but there is considerable controversy about whether that drift is instrumental in speciation^{70,73}. A major problem with founder-effect theories is that they do not seem to work when their verbal assumptions are transformed into mathematical models⁷⁴. Moreover, the observations that inspired such theories are also consistent with a more traditional selectionist model^{73,75}. Biologists have long recognized that species could evolve rapidly on islands or other isolated habitats that provide geographic isolation and novel environments that cause strong natural selection. If reproductive isolation is a byproduct of such selection, one then expects 'adaptive radiation': morphological novelty and rapid speciation in isolated locales.

There are, therefore, two theories explaining the same observations. Adaptive radiation seems a more likely explanation, because its requirement, that reproductive isolation is a simple byproduct of genetic divergence, is probably met in

nature far more often than requirements of founder-effect theories, which postulate complicated mixtures of population structure, demographic fluctuation and genetic interactions. In the end, founder-effect speciation, like sympatric speciation, may be nearly impossible to demonstrate in nature. Although both may occur, they are difficult to distinguish from alternative processes that are better supported by evidence.

Prospects

Despite persistent controversy, evidence still favours the neodarwinian view that species usually arise as the byproducts of evolution in geographically isolated populations. Important parts of this theory, however, rest more on biological plausibility than on hard data. We know little, for example, about how evolutionary divergence causes reproductive isolation. Moreover, we do not know what proportion of reproductive isolation arises from populations adapting to different habitats, and what proportion arises from genetic divergence occurring through random mutation in similar environments. Laboratory experiments could fill these gaps, as well as revealing which isolating factors arise most easily. We do not know which isolating factors initiate speciation in different groups, and which arise only after speciation is complete. Sexual selection may be a major cause of speciation in animals because it is pervasive, leads directly to reproductive isolation, and is associated with many adaptive radiations. Although this form of selection is much discussed, there have been only a few attempts to relate it to speciation^{15,76}. We do not know, for example, what initiates new bouts of sexual selection in geographically isolated populations. The importance of reinforcement is still an open question,

but could be settled through studies of sexual isolation in species with overlapping ranges.

Methods for genetically analysing reproductive isolation have been known for over 50 years, and are technically simple. Until recently restricted to genetically well characterized species, these methods can now be extended to many other groups through molecular mapping. Such studies are important in determining how often reproductive isolation has a simple genetic basis. Without such information, we cannot construct realistic models explaining how speciation results from genetic change in populations. Furthermore, cloning and characterizing speciation genes is now feasible, and may help us build a picture of reproductive isolation from molecular components.

Two of the strongest patterns in evolutionary biology, Haldane's rule and the large effect of the sex chromosomes on postzygotic isolation, still lack wholly convincing explanations. It seems possible that some simple explanation for both phenomena has eluded everyone.

Finally, the study of speciation has been overly preoccupied with attractive theories that are difficult to test, prompting Futuyma's⁷⁷ lament that speciation is "more thoroughly awash in unfounded and often contradictory speculation than any other single topic in evolutionary theory". If ideas such as founder-effect speciation and sympatric speciation are to be taken seriously, their proponents must devise testable predictions to distinguish them from competing ideas. Genetic analysis will certainly have a major role in such tests. □

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Developmental defects of the ear, cranial nerves and hindbrain resulting from targeted disruption of the mouse homeobox gene *Hox-1.6*

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Gene targeting in mouse embryo-derived stem cells has been used to generate mice with a disruption in the homeobox gene *Hox-1.6*. Mice heterozygous at the *Hox-1.6* locus appear normal, whereas *Hox-1.6*⁻/*Hox-1.6*⁻ mice die at or shortly after birth. These homozygotes exhibit profound defects in the formation of the external, middle and inner ears as well as in specific hindbrain nuclei, and in cranial nerves and ganglia. The affected tissues lie within a narrow region along the anteroposterior axis of the mouse but are of diverse embryonic origin. The set of defects associated with the disruption of *Hox-1.6* is distinct from and nonoverlapping with that of the closely linked *Hox-1.5* gene. But both mutations cause loss, rather than homeotic transformation, of tissues and structures.

By analogy with the genes belonging to the *Drosophila* homeotic complex¹⁻³, the vertebrate Hox genes are also believed to specify cell identity along the anteroposterior axis of the embryo⁴⁻⁷. In man and mouse this family of genes contains 38 members, which are distributed in the genome in four linkage groups, Hox 1-4, on four different chromosomes⁸. To determine the genetic function of some of the Hox genes in the mouse, we have used gene targeting in pluripotent mouse-embryo-derived stem (ES) cells to create mice with specific mutations in these genes^{9,10}. Previously it was shown that mice homozygous for a disrupted *Hox-1.5* gene, a member of the Hox 1 linkage group, display developmental defects in several structures derived from a restricted region of the embryo¹¹. Here we describe the phenotype of mice homozygous for a targeted mutation in the closely linked gene *Hox-1.6* and discuss the differences between the phenotype we see and that recently described by Lufkin *et al.*¹² in mice homozygous for a targeted mutation in the same gene.

Disruption of *Hox-1.6* in ES cells and mice

Figure 1a shows the targeting vector pHox-1.6-N/Tk1,Tk2 used to disrupt the *Hox-1.6* gene in ES cells. It contains 11.8 kilobases (kb) of mouse genomic sequence encompassing the *Hox-1.6* locus^{13,14} with the neomycin-resistance (*neo*^r) gene from pMC1neopA¹⁵ inserted into the homeobox domain. Flanking the mouse sequences are the herpes simplex virus thymidine kinase genes TK1 and TK2 (ref. 11). The linearized targeting vector was introduced into ES cells by electroporation, and the cells were then grown in medium containing G418 plus gancyclovir to enrich for transformants that carry the *neo*^r gene targeted into one of the endogenous *Hox-1.6* loci¹⁶. Southern transfer analysis of DNA isolated from G418/gancyclovir resis-

tant colonies showed that 25% of these colonies contained a targeted, disrupted *Hox-1.6* gene. Such an analysis of cell line 1d-5, which contains one copy of the mutated, *neo*^r-disrupted *Hox-1.6* allele and one copy of the normal allele is shown in Fig. 1b.

Six male chimaeric mice were generated by microinjection of 1d-5 cells into C57Bl/6 recipient blastocysts. Test breeding of the chimaeric males to C57Bl/6 females showed that two of these animals transmitted the ES-derived agouti coat colour to their offspring at a frequency of 100% and 20%, respectively. Mice heterozygous for the *Hox-1.6*⁻ mutation are fertile and appear normal. The genotypes of littermates derived from intercrosses of *Hox-1.6*⁻ heterozygotes were evaluated by Southern transfer analyses. Figure 1d shows a representative analysis of such a litter. All three expected genotypes are present. The 1:2:1 segregation of *Hox-1.6* alleles among 210 intercross embryos tested, suggests that *Hox-1.6*⁻/*Hox-1.6*⁻ embryos are not preferentially aborted or resorbed.

Mice containing two defective copies of the *Hox-1.6* gene die at birth or shortly thereafter, with the longest observed period of survival being 3.5 days. The cause of death has not been determined. But as *Hox-1.6*⁻/*Hox-1.6*⁻ mice exhibit hindbrain and cranial nerve defects (see below), physiological functions governed by the hindbrain could be impaired.

Ear compartment defects and distortion of pons

Both inner ear compartments, the middle ear and the external ear are severely affected in *Hox-1.6*⁻/*Hox-1.6*⁻ mice. Frontal sections of mutant mice reveal major defects in the formation of the inner ear compartments, the cochlea and the vestibule, the absence of the cochlear and vestibular nerves and the lack of formation of the middle ear ossicles (Fig. 2). In the external ear, the formation of both the auricle and the external acoustic meatus is distorted (Fig. 3).

The homozygous mutant mice also show distortion of the pons (Fig. 2). This appears to result from an accumulation of fluid in the interaural space late in gestation (evident at E19; Fig. 3d) and after birth (Fig. 2). This compresses the pons. Two observations argue that the altered pons results from mechanical distortion rather than loss of tissue. First, development of the pons appears normal in earlier stages of gestation (E13.5, E15, E18; data not shown). Second, nuclei of the pons do not appear to be absent, but rather displaced (Figs 2c, d).

Defects in cranial nerves and ganglia

Figure 4a shows an E10.5 control embryo immunoreacted with a monoclonal antibody, 2H3, which labels the 155K neurofilament protein (relative molecular mass 155,000)¹⁷. The axons associated with the trigeminal (GV), facial (GVII), vestibulocochlear (GVIII), glossopharyngeal (GIX) and vagus (GX) ganglia are clearly discernible. Figure 4b and c show the 2H3 labelling patterns of two *Hox-1.6*⁻ homozygous littermates. In both mutant embryos the glossopharyngeal and vagus nerves are poorly developed, and the preganglionic connections with

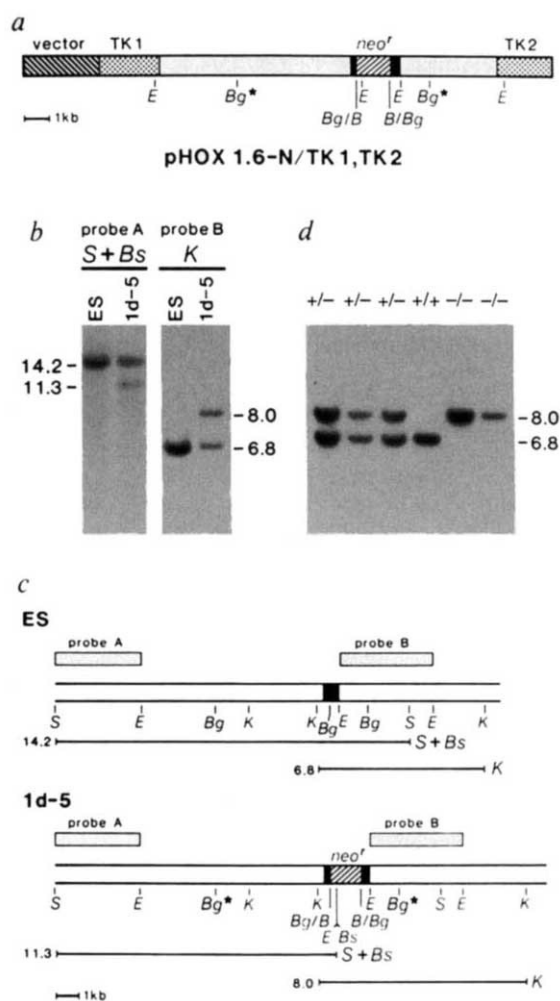


FIG. 1 Southern transfer analysis of ES cell line 1d-5 containing a targeted disruption in the *Hox-1.6* gene (a, b and c) and genotypic analysis of progeny derived from a *Hox-1.6*^{+/+}/*Hox-1.6*^{-/-} intercross (d). a, Structure of the targeting vector, pHOX-1.6-N/TK1,TK2, used to disrupt the *Hox-1.6* gene in ES cells. Two *Bgl*II sites (indicated as Bg*) were eliminated by filling in. b, Southern blot analysis of DNA from the parental ES cell line CC1.2 and the targeted ES cell line 1d-5. The sizes of the DNA fragments are indicated in kb. DNA samples were digested with *Scal* (S) and *Bst*BI (Bs), or *Kpn*I (K), electrophoresed through agarose, transferred to nitrocellulose membrane and hybridized with radioactively labelled probe. Probe A (a 3.4-kb *Scal*-*Eco*RI fragment; see map in c) is a 5' *Hox-1.6* genomic flanking probe, not present in the targeting vector. Probe B (a 3.8-kb *Eco*RI fragment) is a *Hox-1.6* genomic internal probe. In b, the reason that the wild-type band (14.2 kb) is more intense than the mutant band (11.3 kb) in 1d-5 DNA is that the ES cells are grown on feeder cells, whose DNA contributes to the signal of the wild-type band. 1d-5 DNA was also analysed using *Bst*XI and *Bgl*II. The *Bgl*II digestion showed that both modified *Bgl*II sites in the targeting vector were transferred to the target locus (data not shown). The *Bst*XI digestion was carried out as a further check to ensure that no other detectable rearrangements of the target locus occurred during the recombination reaction, either 5' or 3' to the *neo'* insertion. c, Restriction map of the DNA fragments present in the parental and targeted ES cell lines CC1.2 and 1d-5 respectively. d, Genotype, as determined by Southern transfer analysis, of 18-day-old embryos from interbreeding of *Hox-1.6*^{-/-} heterozygous mice. DNA was extracted from tails and analysed. DNA was digested with *Kpn*I and hybridized with internal probe B. The genotype at the *Hox-1.6* locus is indicated above each lane. +/+, Wild type; +/-, heterozygous at *Hox-1.6*; -/-, homozygous for the *Hox-1.6*^{-/-} allele. B, *Bam*HI; Bg, *Bgl*II; Bs, *Bst*BI; E, *Eco*RI; K, *Kpn*I; S, *Scal*. The ES cell line CC1.2 was derived from 129/SV/EV mice³⁰. The source of blastocysts used to generate chimaeric mice was C57Bl/6J mice. The chimaeric mice were bred to C57Bl/6J mice to test for transmission of the mutant *Hox-1.6*^{-/-} allele. The resulting *Hox-1.6*^{-/-} heterozygotes were either interbred or bred to C57Bl/6J mice to maintain the *Hox-1.6*^{-/-} heterozygous colony and to generate *Hox-1.6*^{-/-} homozygotes.

the brain stem are not formed. In the mutant embryo shown in Fig. 4b, the nerves and VII/VIII ganglia are displaced rostrally towards the trigeminal ganglion. This displacement is even more pronounced for the mutant embryo shown in Fig. 4c, resulting in the fusion of GVII/VIII with GV. The nerves associated with GIX and GX also appear to be displaced rostrally along the anteroposterior axis in both embryos. There is a curious asymmetry with respect to the displacement of these nerves/ganglia towards the trigeminal ganglion. It is always more pronounced on the left than on the right side. In six out of six E9.5 and E10.5 embryos that showed the fusion of GVII/VIII with GV, the fusion was observed on the left but not on the right side.

The defects in the cranial nerves depicted in Fig. 4 are also apparent in coronal sections of the same embryos (Fig. 5). For example, the left-side fusion of GVII/VIII with GV is seen in the sections of the *Hox-1.6*^{-/-} homozygote shown in Fig. 5e and f. In these embryos, as well as in other E9.5 and E10.5 embryos, the otocyst as well as the ganglia are displaced rostrally towards the trigeminal ganglion (Fig. 5c-f). Sagittal sections of E9.5 and E10.5 show that the otocyst in the *Hox-1.6*^{-/-} homozygotes, relative to control embryos, is not only displaced rostrally towards the trigeminal ganglion, but also ventrally towards the pharyngeal arches and away from the neural tube (data not shown). The otocyst in all of the *Hox-1.6*^{-/-}/*Hox-1.6*^{-/-} embryos examined is smaller and poorly developed. Further, by E11, the endolymphatic duct, which is normally generated as a dorso-medial outgrowth from the otic vesicle, is not formed in the mutant embryos. But the most pronounced feature of these sections is that the characteristic repeating pattern of hindbrain rhombomeres (Fig. 5a and b) is perturbed in the *Hox-1.6*^{-/-}/*Hox-1.6*^{-/-} embryos (Fig. 5c-f). Instead of having the series of bulges

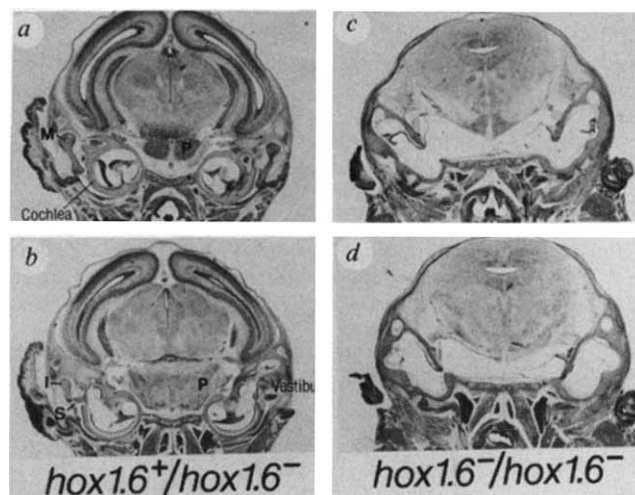


FIG. 2 Comparison of frontal sections (10 microns) prepared from *Hox-1.6*^{-/-} heterozygous (a, b) and *Hox-1.6*^{-/-} homozygous (c, d) newborn mice. Because the stillborn mutant mice have their heads tilted towards their chest, the angle of the plane of section through the brain is not identical in the control (a, b) and mutant sections (c, d). As a result, these sections show tissue from the cerebral hemispheres in the control mouse, but not in the mutant. The sections shown were chosen to illustrate comparable planes in these mice relative to the ears, throat and thorax. The sections from the control mouse (a, b) illustrate the two inner ear compartments, the cochlea and the vestibule. In the cochlea, the spiral organ of Corti and the cochlear nerve are clearly visible. The vestibule and vestibular nerve are shown in b. These sections also show the middle ear ossicles, the malleus (M), the incus (I) and the stapes (S). The corresponding sections from the mutant embryo (c, d) reveal major defects in the formation of both inner ear compartments, the absence of the cochlear and vestibular nerves within these compartments, and the lack of formation of the middle ear ossicles. In addition, the pons (P) of the *Hox-1.6*^{-/-} homozygote is highly distorted. Before sectioning, the mice were fixed in Bouin's reagent and embedded in paraffin. Following sectioning, slides were regressive stained with haematoxylin and eosin³¹. The field width of each figure is 7 mm.

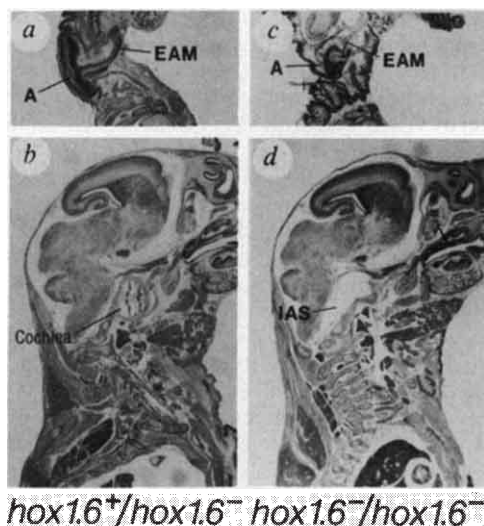


FIG. 3 Comparison of parasagittal sections (10 microns) of E19 *Hox-1.6*^{-/-} heterozygote (a, b) and *Hox-1.6*^{-/-} homozygote (c, d). Sections through the external ear are compared in a and c. Whereas in the control section a well-formed auricle (A) and external acoustic meatus (EAM) are outlined, these structures are greatly distorted in the *Hox-1.6*^{-/-}/*Hox-1.6*^{-/-} mutant mouse (c). In more medial sections of the *Hox-1.6*^{-/-} homozygote (d), not only is the inner ear absent but the interaural space (IAS) is expanded, distorting the adjacent pons. Note that the forebrain and midbrain of the *Hox-1.6*^{-/-} heterozygous and homozygous embryos are indistinguishable. The width of the fields in each figure is 8 mm.

that outline each rhombomere, the walls on both sides of the neural tube of the *Hox-1.6*^{-/-} homozygote are smooth. This feature of the hindbrain has been observed in all mutant embryos examined between stages E9.5 and E11.5.

At all stages of embryogenesis and in newborn mice, the trigeminal ganglion and neural structures rostral to the trigeminal ganglion appear normal in *Hox-1.6*^{-/-} homozygotes (see examples in Figs 3, 4 and 5). By E13.5, the size of GVII and GVIII in mutant embryos is reduced relative to control embryos, and in a number of mutant embryos the facial ganglion was not detectable. Finally, although the inferior ganglion of

the glossopharyngeal and vagus nerves appears fairly normal in E13.5 and E15.5 mutant embryos, the size and shape of the superior ganglia of the same nerves are abnormal. Additionally, the preganglionic connections of the superior ganglia of these nerves with the brain stem and the connections with the inferior ganglia are greatly reduced or absent (data not shown).

Hindbrain defects

At E13.5 through E19, certain hindbrain nuclei are not formed in the mutant embryos. Figure 6a and b shows transverse sections from E18 control embryos of the hindbrain containing the superior olivary complex (SOC) and facial nuclei (FN) respectively. These hindbrain nuclei are either absent or greatly reduced in comparable sections from the *Hox-1.6*^{-/-} homozygous littermates (Fig. 6c and d).

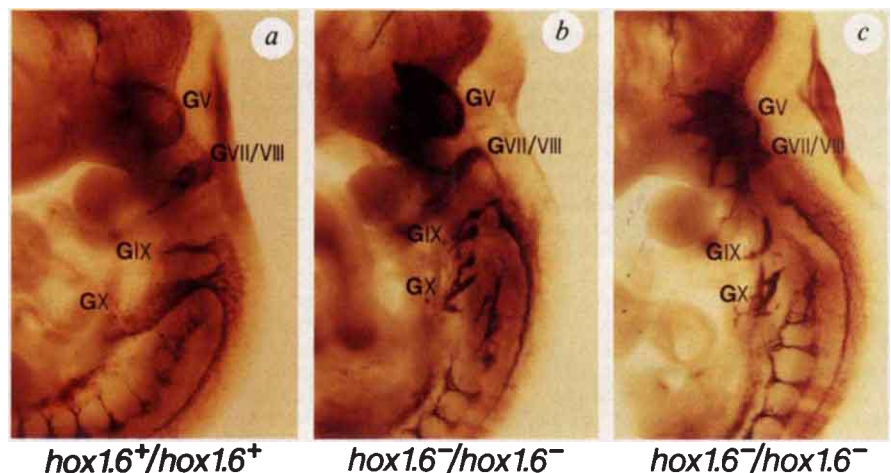
In the hindbrains of E15, E18, E19 and PO *Hox-1.6*^{-/-}/*Hox-1.6*^{-/-} mice, the dorsal and ventral cochlear nuclei as well as the vestibular nuclei were all seen (data not shown). The possibility of quantitative differences in these nuclei between mutant and control mice needs further analysis. The trigeminal nuclear complex appear normal in the *Hox-1.6*^{-/-}/*Hox-1.6*^{-/-} mice. Also, the ambiguous nuclei associated with the glossopharyngeal and vagus nerves were identified in transverse sections from E18 *Hox-1.6*^{-/-} homozygotes and they appear normal.

Discussion

Hox-1.6^{-/-} homozygotes exhibit profound defects in the formation of the external, middle and inner ear. These three contiguous, interacting components of the ear are formed by very different embryonic pathways. Development of the inner ear is first evident as a thickening of the surface ectoderm, the otic placode, which then rapidly invaginates to form the otic vesicle (otocyst). Later in development, the vesicle divides into ventral and dorsal components that differentiate into the cochlea and vestibule, respectively. On the other hand, the ossicles of the middle ear are derived from cartilage of the first and second pharyngeal arches, the malleus and incus being primarily derived from first arch cartilage and the stapes from the second^{18,19}. Finally, the external auditory meatus develops from a dorsal portion of the first pharyngeal cleft, whereas the auricle develops from six mesenchymal proliferations located at the ends of the first and second pharyngeal arches^{18,19}. Despite the difference in embryonic derivation of these components, *Hox-1.6* seems

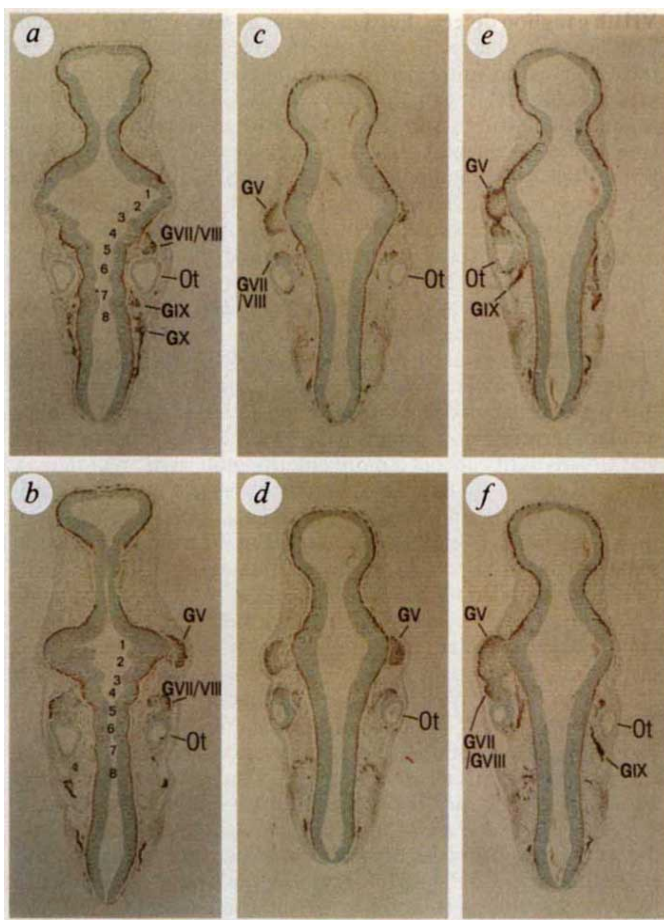
FIG. 4 E10.5 mouse embryos immunoreacted with a monoclonal antibody against the 155K neurofilament protein. a, Control *Hox-1.6*^{-/-}/*Hox-1.6*^{-/-} embryo; b and c, two mutant *Hox-1.6*^{-/-}/*Hox-1.6*^{-/-} embryos. a, Control embryo showing axons of the trigeminal ganglion (GV), the facial ganglion (GVII), the vestibulocochlear ganglion (GVIII), the glossopharyngeal ganglion (GIX) and the vagus ganglion (GX). b and c, Two mutant embryos showing poorly developed glossopharyngeal and vagus nerves and absence of preganglionic connections with the brainstem. Also in the mutant embryos, ganglia GVII through GX appear to be rostrally displaced towards the trigeminal ganglion (GV). The displacement is particularly marked in the *Hox-1.6*^{-/-}/*Hox-1.6*^{-/-} embryo shown in c, resulting in the fusion of GVII/GVIII with GV.

METHODS. Embryos were processed according to a protocol from A. Lumsden (personal communication) and fixed in 3.5% formaldehyde/10 mM phosphate buffer (pH 7.4), then washed three times with PBS for 60 min. Endogenous peroxidase activity was blocked by an overnight incubation at 4 °C with 0.05% hydrogen peroxide, 1% Triton X-100, and 1% fetal bovine serum-PBS. Embryos were washed three times with 1% Triton X-100, 1% normal goat serum-PBS (WS) for 60 min and incubated for 4 days at 4 °C with the monoclonal antibody 2H3 (ref. 16) diluted 1:1 with WS; they were then washed three times with WS for 60 min and incubated overnight at 4 °C with goat antimouse-IgG conjugated with horse-



radish peroxidase (diluted 1:200 with WS). After washing as before, the embryos were incubated with 0.5 mg ml⁻¹ diaminobenzidine in 50 mM Tris-HCl, pH 7.4, for 3 h at 4 °C in the dark, followed by a 10-min incubation at room temperature with diaminobenzidine containing 0.018% hydrogen peroxide. The reaction was terminated by three washes with PBS (60 min each). The width of each field shown in a-c is 2 mm.

FIG. 5 Comparison of coronal sections (10 microns) of E10.5 embryos immunoreacted with the 155K neurofilament antibody. *a, b*, Control *Hox-1.6⁺/Hox-1.6⁻* embryo; *c-f*, *Hox-1.6⁻/Hox-1.6⁻* mutant embryos. Sections shown in *a, c* and *e* are more dorsorostral than the corresponding sections shown in *b, d* and *f*. These sections were derived from the embryos shown in Fig. 4 and counterstained with methyl green. Note that the rhombomeric pattern of the hindbrain shown in the control embryo (*a, b*) is perturbed in the mutant embryos (*c-f*). Rather than showing the characteristic bulges that outline each rhombomere, the wall on both sides of the neural tube is smooth in the mutant embryos. GIX and GX are poorly developed in the mutant sections. Fusion of GVII/VIII with GV can be seen on the left side of the mutant embryo shown in *e* and *f*. In addition to these ganglia being displaced rostrally towards the trigeminal ganglion, the otocyst (Ot) is also displaced rostrally. The width of each frame is 1.7 mm. The numbers 1–8 in *a* and *b* indicate the approximate positions of rhombomeres 1–8.



to be required for the formation of all of them. *Hox-1.6* could be independently involved in execution of each of these pathways, or there could be a domino effect, with defective *Hox-1.6* regulation of an early component (for example, the inner ear) leading to malformation of a later component, such as the middle ear. Simple versions of the latter hypothesis are, however, contradicted by the existence of many mutations in the mouse and man that can drastically affect the development of the inner ear without affecting development of the middle and external ear²⁰. In fact, no previously described mutation in the mouse or man simultaneously affects the formation of inner, middle and external ear. As mentioned, the inner ear is derived from the otocyst. Studies in several species²¹ have concluded that development of the membranous labyrinth requires continued passage of inductive signals between the otocyst and the adjacent neural tube. In E9.5 and E10.5 *Hox-1.6⁻/Hox-1.6⁻* embryos, we consistently observed rostral and ventral displacement of the otocyst. Such displacement could interfere with the reception of inductive signals between the otocyst and the neural tube.

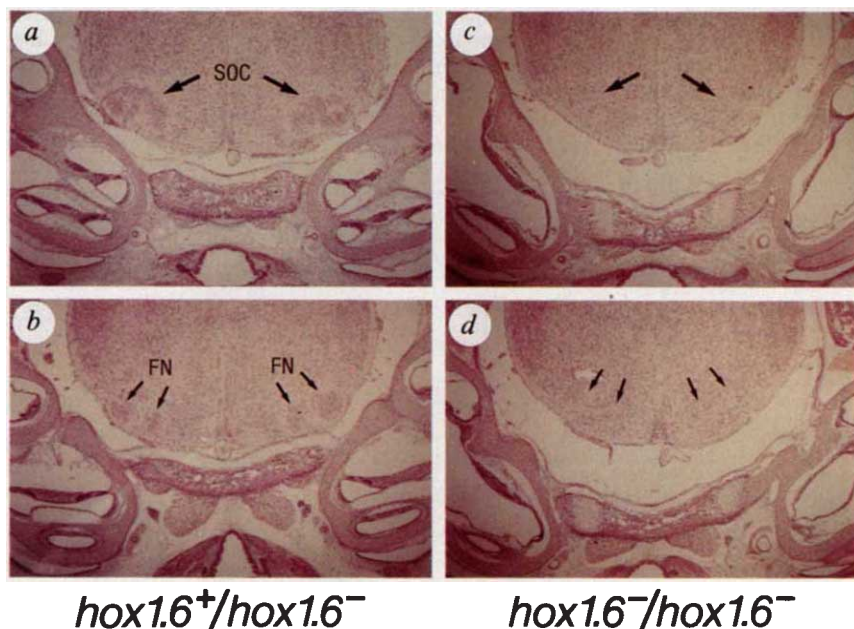
Hox-1.6⁻/Hox-1.6⁻ mice also exhibit defects in the hindbrain and associated cranial nerves and ganglia. Particularly prominent is the severe reduction or absence of connections between the glossopharyngeal and vagus nerves/ganglia with the brainstem. This defect is observed as early as E9.5 as a lack of formation of preganglionic connections between these ganglia and the brainstem. Also at E9.5 rostral displacement of GVII through GX towards the trigeminal ganglion is evident, often

hox1.6⁺/hox1.6⁻

hox1.6⁻/hox1.6⁻

resulting in the fusion of GVII/VIII with GV. Later in embryogenesis (E13.5 onward), loss of tissue associated with the facial and vestibulocochlear ganglia is observed. This loss could result from either a lack of cell proliferation or atrophy of these tissues. As GVIII is largely of otic placode origin²², defects affecting the development of this placode could be expected to result in

FIG. 6 Comparison of transverse sections (10 microns) of E18 mouse embryos prepared from a control *Hox-1.6⁺* heterozygote (*a, b*) and a mutant, *Hox-1.6⁻* homozygote (*c, d*). Sections *b* and *d* are more caudal than those of *a* and *c*. The nuclei of the superior olivary complex (SOC), which are involved in relaying signals from the cochlear nuclei to the inferior colliculus, and the facial nuclei (FN) are shown in the control sections *a* and *b* respectively. These nuclei were not visible in the comparable or adjacent sections from the mutant embryo. At high magnifications a few cells resembling facial motor neurons were seen in the *Hox-1.6⁻/Hox-1.6⁻* section that may represent the remnants of the facial nuclei. Similar patterns were evident in transverse sections from E15 and E19 control and mutant embryos. The width of each field shown in Fig. *a-d* is 3.5 mm.



hox1.6⁺/hox1.6⁻

hox1.6⁻/hox1.6⁻

VIIIth ganglion defects. Further, the target tissue for the cochlear and vestibular nerves is not formed, so it is not surprising that those nerves do not enter their respective inner ear compartments. The facial ganglion (GVII) is derived largely from cephalic neural crest, which arises adjacent to rhombomere 4 (refs 22–26). A phenotypic effect on this neural crest population by the *Hox-1.6*⁻ mutation is consistent with the reported anterior expression for *Hox-1.6* (refs 12, 27, 28). It is interesting that all neurological defects associated with disruption of *Hox-1.6* are present within a narrow region bounded by rhombomeres 4 through 7 (refs 25, 26).

Careful comparison of *Hox-1.6*⁻/*Hox-1.6*⁻ and *Hox-1.5*⁻/*Hox-1.5*⁻ mice¹¹ has revealed no defects in common between the two. Each mutation gives rise to a complex but distinct set of abnormalities. Each set, moreover, is restricted to a limited region along the anteroposterior axis of the mouse embryo, the *Hox-1.6*⁻ defects being, on the whole, in structures and tissues derived from a more anterior embryonic region than those of *Hox-1.5*⁻/*Hox-1.5*⁻. This is consistent with the more anterior limit of expression of the *Hox-1.6* gene. As with *Hox-1.5*, the anterior limit of expression rather than the overall expression pattern, is a better predictor of where mutation of the gene will be expressed phenotypically.

The primordia responsible for the formation of the inner ear, the hindbrain nuclei, the cranial nerves and the associated ganglia affected by disruption of *Hox-1.6* are located in a region of the early embryo encompassing rhombomeres 4–7 (refs 28, 29). The defects in the formation of the middle and external ears may be exceptions. Do these defects represent primary defects in pharyngeal arch-1 and/or associated mesenchymal neural crest? The formation of the auricle requires the intimate interaction between arch-1 and arch-2 mesenchyme, so an arch-2 defect could account for the malformation of this structure. But it is much more difficult to argue that an arch-2 defect could lead to the complete absence of the malleus and the incus because they are derived primarily, if not entirely, from arch-1-associated neural crest^{18,19}. Should the lack of formation of these ossicles in the *Hox-1.6*⁻/*Hox-1.6*⁻ mice reflect a primary defect in arch-1-associated neural crest, then a paradox exists. Based on experiments that follow migration of neural crest cells in chick embryos, Lumsden *et al.*²⁹ have proposed that rhombomere 2-associated cephalic neural crest cells migrate into arch 1, rhombomere 4-associated neural crest cells migrate into arch 2, and so on. As the expression pattern of *Hox-1.6* is presumed not to extend into rhombomere 2, we would not anticipate an arch 1-associated phenotype in *Hox-1.6*⁻/*Hox-1.6*⁻ mice. There are many ways to reconcile this apparent contradiction. One possibility is that the *neo'* gene inserted into the *Hox-1.6* homeobox is influencing the expression pattern of neighbouring *Hox* genes. If this were the case, that expression does not produce a gain of function phenotype, as *Hox-1.6*⁻ heterozygotes show

no phenotype. Heterozygous mice were examined as carefully as homozygous mice as they were most frequently used as controls. Another possible explanation for the apparent contradiction is that although the majority of rhombomere 4-associated neural crest migrates into the second pharyngeal arch, a subpopulation may migrate into arch 1. A third possibility is that the position of the anterior expression boundary of *Hox-1.6*, particularly during the critical period before the formation of the rhombomere boundaries, has not yet been fully defined. There may be subpopulations of cells that express *Hox-1.6* but are positioned ahead of the visible anterior boundary.

Lufkin *et al.* have also reported on the phenotype of mice containing targeted disruptions in *Hox-1.6* (ref. 12). Their *Hox-1.6*⁻ phenotype is similar to ours but there are interesting differences. In both cases *Hox-1.6*⁻/*Hox-1.6*⁻ mice have defects in the inner ear compartments; however, the extent of the defects appears more extreme in the mice shown in Figs 2, 3 and 6. Lufkin *et al.* did not report that *Hox-1.6*⁻/*Hox-1.6*⁻ mice contain middle and external ear defects, whereas we found complete loss of the middle ear ossicles and severe distortion in the formation of the external ear. Similarly, the neuropathology of the respective *Hox-1.6*⁻/*Hox-1.6*⁻ mice display similarities and differences. In both cases there was a loss of connections between the brainstem and the glossopharyngeal and vagus nerves. But Lufkin *et al.* did not report the rostral displacement of the cranial ganglia GVII through GX towards the trigeminal ganglion described here. This feature may be more apparent early in embryogenesis than at later stages. Also they did not report the displacement of the otocyst nor the absence of the nuclei of the superior olivary complex. Although we examined eight E9.0–E9.5 embryos, we did not observe a delay in the closure of the neural tube described by Lufkin *et al.* On the other hand, they reported that *Hox-1.6*⁻ homozygotes have normal rhombomere morphology, whereas we consistently observe that between E9.0–E11.0 the rhombomeres in the mutant hindbrain do not exhibit the characteristic bulges that outline each rhombomere. The reported defects in the basioccipital bone, as well as in the bones and cartilages associated with the inner ear, are similar in the two mutant mice (data not shown).

Some of the differences in the phenotype could arise from the differences in the mutant alleles of *Hox-1.6* created by the two groups. Two transcripts are generated from the *Hox-1.6* locus as a result of alternative splice sites within the *Hox-1.6* gene^{13,14,28}. One transcript directs the synthesis of the entire *Hox-1.6* protein product, including the homeobox domain. The second transcript may direct the synthesis of a truncated protein with the same NH₂ terminus but lacking the homeobox domain. The *Hox-1.6*⁻ mutant allele made by Lufkin *et al.*¹² is predicted to eliminate the synthesis of both transcripts, whereas our mutant allele should disrupt the synthesis of only the intact homeobox-containing protein. □

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Estimating redshifts for γ -ray bursts

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RECENT observations from the BATSE experiment on the Gamma Ray Observatory^{1,2} demonstrate that the number of weak γ -ray bursts is smaller than expected for a uniform distribution of source distances, and that the distribution of weak bursts is isotropic. This suggests that the sources are at cosmological, rather than intragalactic, distances^{3,4}. A number of possible tests for estimating the distances to γ -ray bursts have been discussed³, but all are difficult to implement in practice. There is therefore no further evidence at present to support a cosmological distance to the bursters. Here I describe a test of distances that might be done with the BATSE data, which is equivalent to the formulation of the Hubble diagram for galactic redshifts. If the typical redshift of γ -ray bursts is of the order of unity, the weakest bursts should have softer spectra than the strongest. There is some evidence for such a correlation in the BATSE data⁵. If confirmed, this would provide the first spectroscopic indication that the burst sources lie at cosmological distances. There is also evidence that the redshift effect has been detected in the duration of a subset of the BATSE bursts⁶. If confirmed, this would be evidence that not only the photons but also the light curves are stretched by the cosmological redshift.

For a classical Hubble diagram, the redshift to the source has to be measured using well defined spectral lines. This cannot be done for γ -ray bursts. There are claims that annihilation and cyclotron lines were detected in some events⁷, but those lines were too elusive to be useful for redshift measurements. The power-law continuum is featureless, and therefore it is probably impossible to obtain redshifts from individual spectra. It may, however, be possible to measure statistical redshifts, as the observed spectra do not follow perfect power laws.

A spectrum of a γ -ray burst is sometimes described as a broken power law, with a break at ~ 1 MeV, but that description is not unique⁶. It seems, however, that the slope changes in the

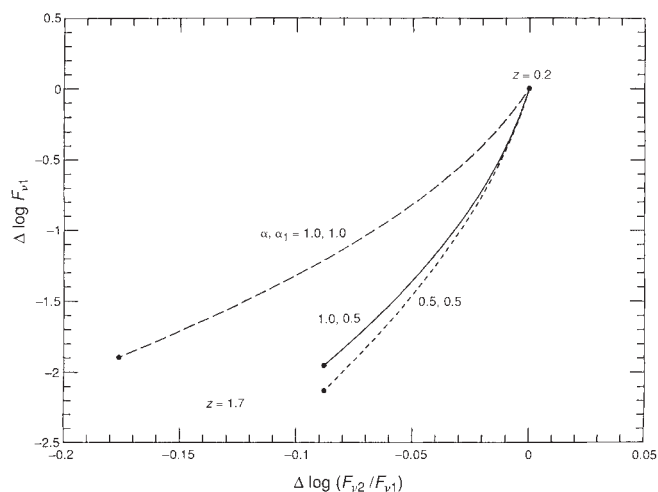


FIG. 2 'Colour-luminosity' diagram for 'standard candle' sources at two fixed frequencies: ν_1 and ν_2 . The differences are with respect to a source at redshift $z=0.2$, taken to correspond to the strongest bursts detected by BATSE so far. The three lines correspond to three sets of values of the spectral parameters α and α_1 , as defined by equation (1). The redshift varies along the lines from $z=0.2$ to $z=1.7$, as indicated. The weakest bursts detectable by BATSE may be at the redshift $z=1.7$.

same direction in all spectra above ~ 10 keV: we have $d\alpha/d\log\nu < 0$, where α is the spectral index, defined as $\alpha \equiv d\log(\nu F_\nu)/d\log\nu$. This index is in the range $0.5 < \alpha < 1.2$ for photon energies $30 \text{ keV} < E_\gamma < 500 \text{ keV}$, and varies from burst to burst⁶.

The simplest form of a spectrum with a slowly changing slope is

$$\log(\nu L_\nu) = C + \alpha \log \nu - 0.5\alpha_1(\log \nu)^2, \quad \alpha > 0, \quad \alpha_1 > 0 \quad (1)$$

where C is a constant and L_ν is the monochromatic luminosity of the source. The logarithmic slope is given as

$$\frac{d\log(\nu L_\nu)}{d\log\nu} = \alpha - \alpha_1 \log \nu \quad (2)$$

and the spectrum peaks reaches maximum at $\nu = \nu_m$ given as

$$\log \nu_m = \alpha / \alpha_1 \quad (3)$$

Real spectra are no doubt more complex, but equation (1) is sufficient for the point being made here.

The simplest cosmological picture is that of a non-evolving population of γ -ray bursters, with constant burst rate per comoving volume and comoving time, in a Friedman universe with $\Omega = 1$ and $\Lambda = 0$. All bursters are assumed to be standard candles with identical spectra given by equation (1). The flux distribution observed on Earth varies with redshift according to

$$\nu F_\nu = \frac{(1+z)\nu L_{(1+z)\nu}}{4\pi d_L^2(z)} \quad (4)$$

where z is the source redshift and

$$d_L(z) = \frac{2c}{H_0} [(1+z) - \sqrt{1+z}] \quad (5)$$

is the luminosity distance⁸. Examples of the model flux distributions are shown in Fig. 1 for standard candle sources at redshifts $z=0.2, 0.5, 1.0$ and 1.7 . In the simplest cosmological model⁴ the redshifts 0.2 and 1.7 correspond to the strongest and the weakest γ -ray bursts detected so far with the BATSE experiment.

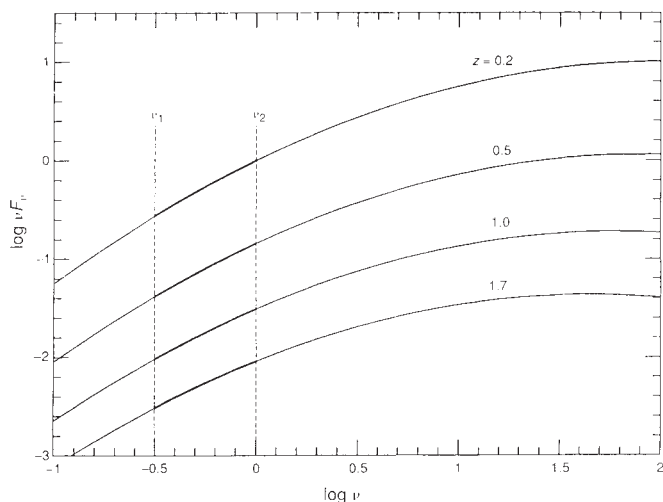


FIG. 1 Spectra of 'standard candles' as given by equation (1), with $\alpha=1.0$, $\alpha_1=0.5$, shown for sources placed at four redshifts: $z=0.2, 0.5, 1.0$ and 1.7 . The observations are made at two frequencies: ν_1 and ν_2 , indicated by the two vertical dashed lines. The spectral slope between these two frequencies decreases with increasing redshift of the source, and the overall intensity is reduced.

Let us consider an instrument capable of measuring flux at two different frequencies, ν_1 and ν_2 . Using our cosmological model we may calculate how F_{ν_1} and F_{ν_2} vary with the source redshift. The redshift itself is not observable, but we can make a 'colour-luminosity' diagram: $\Delta \log F_{\nu_2}/F_{\nu_1}$ against $\Delta \log F_{\nu_1}$, where the differences are given with respect to a fiducial source at $z=0.2$. Examples of such colour-luminosity relations are shown in Figure 2 for a few choices of the two spectral parameters, α and α_1 , and for $\log \nu_2/\nu_1 = 0.5$. The trend is always the same: the weaker sources have softer spectra. This is so because the spectra were assumed to be of the type given by equation (1), in which the first derivative of the slope is negative. The softening of the spectra is more noticeable when the frequency baseline ν_2/ν_1 is large, and the spectral index varies rapidly (α_1 is large). In fact, if the hardness of the spectrum is defined as $\log(F_2/F_1)$, then its variation with the source redshift is proportional to $\alpha_1 \times \log(\nu_2/\nu_1)$. The larger the spectral baseline, the larger the 'colour' change due to redshift.

The larger the limiting redshift for the sample, the stronger the 'colour' change. The BATSE is the first experiment that is sensitive enough to see the 'edge' of the distribution; that is, to reach $z \sim 1$, assuming that the cosmological model provides the correct interpretation of the 'edge'. The spectra of BATSE sources are therefore the first that may show the expected correlation between the spectral hardness and the burst intensity.

The real bursters are not likely to be standard candles, and their spectra are observed to have a large range of slopes⁷, for the strong as well as for the weak bursts. Therefore, instead of a single line, as in Fig. 2, a lot of scatter is expected, and the trend can only be detected statistically. This correlation seems to be present in the spectra of BATSE bursts, with the weaker bursts being somewhat softer⁵. It is difficult to judge at present

whether the effect is real. If confirmed, this correlation would provide a spectral indication that the weak bursts are redshifted with respect to the strong ones. The effect may be detectable even without absolute calibration of the spectra. Once the spectra are well calibrated, and the values of α and α_1 are measured, it will be possible to estimate the redshift of the sources using the empirical equivalents of Fig. 2.

If the weakest BATSE bursts are at a redshift $z \approx 1.7$, then their light curves (time histories) should be stretched in time by a factor $(1+z)$ as compared with the strongest bursts. It is well known that burst durations cover many orders of magnitude, so it is not clear at all if a relatively small factor like 2.7 is detectable. A small subset of 'simple' single-peaked bursts as observed by BATSE⁶ does, however, show the expected correlation: the rise times of the weakest bursts are longer, on average, than the rise times of the strongest bursts. It is too early to claim that this is the redshift effect. All possible instrumental effects have to be understood first. If confirmed, the correlation between rise time and peak intensity will indicate that the bursters are at cosmological distances. $\square$

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Gamma-ray bursts in the galactic halo

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THE angular and luminosity distributions of the γ -ray bursts observed by the BATSE instrument, on the Gamma Ray Observatory satellite, cannot be explained by sources confined to the galactic plane¹. Instead the observations are consistent with a nearly isotropic source density that falls with distance. Although this permits a cosmological origin, it also permits an origin in the halo of our Galaxy, preserving some aspects of models in which galactic neutron stars are the sites of the bursts. I show here that significant isotropy can be achieved with a spherically symmetric halo model if it extends out beyond 100 kpc. Large halo core radii enhance isotropy, although consistency with observation is possible for core radii as small as 5 kpc if the halo radius is sufficiently large. The intrinsic luminosity distribution of γ -ray bursts must be treated as a free parameter to fit the observations. If γ -ray bursts are from the halo, they are likely to be old population II neutron stars, because models based on pulsars escaping from the galactic plane have strong anisotropies.

The leading model for γ -ray bursts is the emission of radiation in the magnetosphere of a neutron star². Their rapid rise time and 10-ms variability suggest a source smaller than 10^8 cm, and the numerous bursts with fluxes above 10^{-5} erg cm⁻² s⁻¹ give luminosities of order 10^{38} erg s⁻¹ at 300 pc, the luminosities associated with accreting neutron stars. Additional support for this model comes from the observation of spectral features in the Konus data that can be interpreted as redshifted electron-

positron annihilation lines and cyclotron resonances from a magnetic field of $\sim 4 \times 10^{12}$ G (ref. 3). Ginga provided stronger evidence by observing in three separate bursts two spectral features interpreted as the first two cyclotron resonances^{4–6}. Assuming a galactic origin, many astrophysicists expected a concentration of burst sources along the galactic plane similar to the spatial distributions of X-ray binaries and radio pulsars.

The BATSE instrument of the Gamma Ray Observatory (GRO) satellite is admirably suited to find the distribution of γ -ray bursts on the sky⁷. The preliminary results, based on 117 γ -ray bursts, indicate that the sources are isotropic to the statistical error of a random distribution¹. The average cosine of the

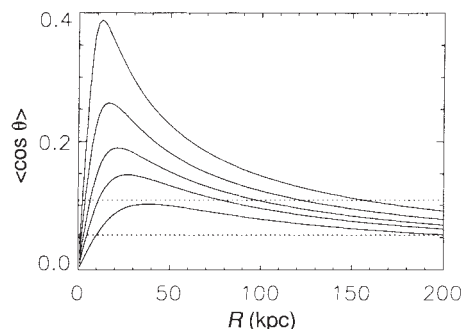


FIG. 1 Plot of $\langle \cos \theta \rangle$, the average cosine of the burst angle relative to the Galactic Centre for the galactic halo model, as a function of the maximum observable distance R . The curves, from top to bottom, are for halo core radii of 4.25, 8.5, 12.75, 17 and 25.5 kpc. The dotted lines mark $\langle \cos \theta \rangle = 0.054$ and 0.108, at one and two standard deviations from zero for the current observations.

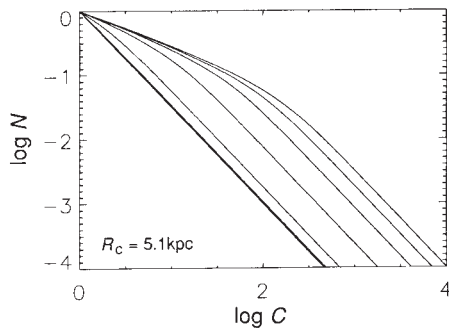


FIG. 2 Plot of $\log N$ against $\log C$. The number of bursts producing a maximum count rate greater than C is plotted for a core radius of 5 kpc for the galactic halo. The units are arbitrary. From top to bottom, the values of the maximum observable distance R are 170, 127.5, 85, 42.5, 17, 8.5 and 4.25 kpc, with the last two curves almost identical.

angle between the source and the Galactic Centre is $\langle \cos \theta \rangle = 0.008 \pm 0.054$ (the published standard deviation¹ of 0.071 is in error; G. J. Fishman, personal communication). There is no evidence of either the galactic plane or of the Galactic Centre. These results show that the weak bursts are as isotropic as the previously observed strong bursts^{8,9}. In addition, the average ratio of the volume delineated by a source (that is, enclosed within a sphere extending to that source) to the maximum volume observed is $\langle V/V_{\max} \rangle = 0.34 \pm 0.03$ (ref. 1), which is smaller than the 0.419 ± 0.18 found by the Signe experiment¹⁰. If the sources were uniformly distributed through space, one would expect 0.5; that the observed value falls below this suggests that the source density decreases with distance. The conclusion forced on us if we assume a non-Solar System origin is that these objects are either in an extended galactic halo¹¹ or are at cosmological distances¹². Below I show that the present results cannot exclude a halo origin, although the observations place tight constraints on the allowable models.

The galaxy has a dark halo extending to 50 kpc and, if the interpretation of the magellanic stream is correct, extending beyond 100 kpc (ref. 13). Models for the halo generally assume a mass distribution of $\rho \propto (r_c^2 + r^2)^{-1}$, where r_c is the halo core radius, for a radial distance from the Galactic Centre of $r < r_1$, where r_1 is the outer radius of the halo. The mass density drops to zero for $r > r_1$. Assuming that the peak flux from γ -ray bursts drops below background before r approaches r_1 , and that the sources are distributed as the halo mass, one finds that for $r_c = 5$ kpc, the sources are highly skewed toward the Galactic Centre if the maximum observable distance R is ~ 10 kpc, but are relatively isotropic if R exceeds 50 kpc (ref. 12). As r_c increases, the bursts become more isotropic for all R . Figure 1 shows $\langle \cos \theta \rangle$ as a function of maximum observable distance for several values of r_c . This calculation assumes that γ -ray bursts have only one peak luminosity. The value $\langle \cos \theta \rangle = 0.1$ is within two standard deviations of the BATSE result. This value is consistent with core radii of 8.5 kpc and greater in Fig. 1 for $R > 100$ kpc, and is consistent with $r_c = 4.25$ kpc for $R > 150$ kpc. The galactic-halo model gives $\langle \cos \theta \rangle \geq 0.05$, and is therefore invalid if a statistical sample of bursts has $\langle \cos \theta \rangle = 0$ with a standard deviation much less than 0.05.

The r^{-2} dependence of the halo beyond the core radius r_c leads to $d \log N/d \log C = -\frac{1}{2}$, where C is a photon count rate and N is the number of γ -ray bursts with a peak photon count rate above C . If the γ -ray sources are located nearby so that their distances are much less than the halo length scale, one finds $d \log N/d \log C = -\frac{3}{2}$. Figure 2 shows $\log N$ against $\log C$ for a uniform-luminosity burst distribution with maximum observable distances R of 4.25, 8.5, 17, 42.5, 85, 127.5 and

170 kpc. For $R < 17$ kpc, $d \log N/d \log C \approx -\frac{3}{2}$, but for $R > 42.5$ kpc, $d \log N/d \log C$ is $-\frac{1}{2}$ for the weakest bursts, and breaks to $-\frac{3}{2}$ as the burst strength increases. These larger distances therefore produce a relatively isotropic distribution with a gradient $d \log N/d \log C$ that deviates from $-\frac{3}{2}$. A multi-luminosity burst population can further smooth the break between $-\frac{1}{2}$ and $-\frac{3}{2}$ at the cost of a larger $\langle \cos \theta \rangle$, so $d \log N/d \log C$ is a measure of both the spatial distribution and the peak luminosity distribution of γ -ray bursts. Equivalently, the value of $\langle V/V_{\max} \rangle$ must fall in a range between $\frac{1}{4}$ and $\frac{1}{2}$, with the precise value dependent on the peak luminosity distribution. Allowing for a range of luminosities lowers Paczyński's restriction of $r_c > 17$ kpc (ref. 12) on the size of the galactic halo's core. A maximum count-rate distribution that is a $C^{-0.7}$ power law up to a cut-off value C_{co} gives the observed value of $\langle V/V_{\max} \rangle \approx 0.35$ while simultaneously giving $\langle \cos \theta \rangle \leq 0.1$ for $R > 200$ kpc and $r_c \geq 8.5$ kpc. For $\langle \cos \theta \rangle < 0.15$, one can have $\langle V/V_{\max} \rangle \approx 0.35$ for $R > 100$ kpc and $r_c \leq 5.0$ kpc. This is shown in Fig. 3, where the solid lines are contours of constant R and the dotted lines are contours of constant r_c . The plotted region shifts to the right as the power-law index δ of the count rate distribution function $C^{-\delta}$ decreases. A similar shift occurs if the source distribution falls less rapidly than r^{-2} . The BATSE results are at present consistent with a galactic-halo origin of γ -ray bursts for reasonable values of the galactic core radius. But with a $2-3\sigma$ deviation from the observed value, the constraints on the model are tight. A test of the galactic-halo model is evident from Fig. 3: the value of $\langle \cos \theta \rangle$ against $\langle V/V_{\max} \rangle$ is strongly dependent on the maximum observable distance, which is equivalent to a dependence on the minimum count rate in the observations.

The galactic-halo model places an upper limit on the burst luminosity. BATSE can detect γ -ray burst fluxes down to 10^{-7} erg cm⁻² s⁻¹. For a burst to fall below the background inside a 200-kpc galactic halo, the peak burst luminosity must be less than 4×10^{41} erg s⁻¹. A similar number can be derived for γ -ray bursts from the halo of nearby spiral galaxies. For M31, the luminosity must be greater than 2×10^{42} erg s⁻¹ to be seen as a burst excess over a circle of radius 15° (ref. 14). This suggests that the peak luminosity function must drop by at least an order of magnitude between these two luminosities.

The shape of the halo is unknown, so the most conservative approach is to assume that halo neutron stars have an isotropic distribution about the Galactic Centre and that our offset from

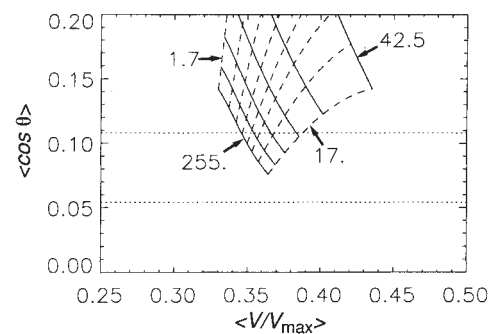


FIG. 3 Plot of $\langle \cos \theta \rangle$ against $\langle V/V_{\max} \rangle$. The relationship between these two values for different halo core radii and maximum observable burst distances is plotted for a power-law burst count rate of index 0.7. The solid lines are calculated for variable core radius and fixed values of maximum observable distance; from upper right to lower left the maximum observable distances are 42.5, 85, 127.5, 170, 212.5 and 255 kpc, all assuming a solar distance from the Galactic Centre of 8.5 kpc. The dashed curves from the top left curve to the bottom right curve are calculated for constant galactic halo core radii of 1.7, 3.4, 5.1, 6.8, 8.5, 10.2, 12.75 and 17 kpc. The dotted curves represent one and two standard deviations away from $\langle \cos \theta \rangle = 0$ of the BATSE results.

the Galactic Centre is the only source of anisotropy. Although physically unjustified, this model produces the minimum likely value of the dipole moment relative to the Galactic Centre, as any asymmetry in the galactic halo will add to the size of the dipole moment unless one assumes a fortuitous cancellation of the offset dipole by the asymmetry. The halo neutron stars may be the remnants of population II stars. A second possibility is that neutron stars are ejected into the halo from the galactic disk at a high velocity, as is indicated by proper motion and interstellar scintillation studies^{15,16}, which find velocities averaging $\sim 150 \text{ km s}^{-1}$ and ranging beyond 400 km s^{-1} relative to the galactic disk velocity, and is suggested by the high-velocity interpretation¹⁷ of pulsar PSR1757-24. In such a model, the comparison of the spatial distribution of γ -ray bursts to the distribution of pulsars is invalid, because the pulsar distribution is determined by the short pulsar lifetime of 10^7 yr (ref. 18). If a significant number of neutron stars are injected below the escape velocity, which is likely from current observations, the distribution function will be quite different from the assumed dark halo distribution, producing considerable dipole and quadrupole moments¹⁹. If most injected neutron stars have velocities far above the escape velocity of the galactic halo, in which case the catalogues of pulsar velocities are grossly incomplete, then these stars will have a spatial distribution that falls roughly as

r^{-2} far from the galactic disk and an asymmetric core enveloping the galactic disk. Reliance on neutron stars in the galactic plane requires extreme models for populating the galactic halo. $\square$

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Collimation of astrophysical jets by inertial confinement

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MANY astrophysical objects, from young stars, Herbig-Haro objects and planetary nebulae up to active galactic nuclei, can be very simply modelled as isotropic sources of high-energy tenuous gas embedded in dense toroidal clouds. Here we describe numerical simulations showing how such an arrangement can in general circumstances give rise to a well collimated jet, as is observed in many of these systems. Our model is a two-dimensional generalization of the interacting-winds description of planetary nebulae. Where the two winds come into contact, a discontinuity is formed, which is dragged out by the fast outflowing gas into a chimney along the polar axis. High-energy gas rushes up this channel and flows out around the top, creating a hot backflow which keeps the chimney in place. The inner shock, enclosing the source of the fast wind, also aids in collimation, and ionization cones such as those observed in active galactic nuclei may also form.

In our work on the hydrodynamics of aspherical planetary nebulae (PNe), we have come across a mechanism for the formation of hydrodynamic jets which is very effective, and which easily extends to situations other than PNe. Planetary nebulae are bubbles blown by a tenuous, fast stellar wind into a dense, slow, fossil red-giant envelope. Balick¹ generalized this model to two dimensions to explain the morphologies of aspherical PNe. Observations indicate that the envelope has cylindrical symmetry, and that the density is higher at the equator than at the poles. The PN morphology and its evolution is then purely a consequence of the mass distribution in the envelope and its interaction with the fast stellar wind.

Analytical²⁻⁵ and numerical⁶⁻⁸ studies of the hydrodynamics of this model have yielded results that comfortably fit the many

morphologies of PNe. Here we argue that the model has broader astrophysical applications.

The details of our hydrodynamic method^{6,8} have been discussed elsewhere. We use spherical coordinates (r, θ, ϕ) with $\partial/\partial\phi = 0$. As initial conditions in the envelope, we took the family of disk-toroids described by³⁻⁵

$$\rho(\theta, r) = \frac{\rho_*}{A(\theta)} \left(\frac{r_0}{r} \right)^2 \quad (1)$$

$$A(\theta) = 1 - \alpha \left(\frac{e^{\beta \cos 2\theta} - 1}{e^{-2\beta} - 1} \right) \quad (2)$$

Here α describes the equator-to-pole contrast ('e/p ratio'), and β governs the decrease of the density with polar angle θ . This function represents plausible models of gaseous disks. Its isodensity contours range from ellipsoidal ($\beta \rightarrow 1$) through quasi-toroidal ($\beta > 3$, $\alpha \rightarrow \infty$) to annular ($\beta \approx 1$, $\alpha > 20$). Narrow funnels can also be constructed ($\beta > 10$, $\alpha > 5$).

We have run more than 50 simulations. Of these, we show one case which is representative for the jet formation mechanism we discovered. The initial conditions are as follows (all quantities are given at a reference position on the symmetry axis, $\theta = 0$, radius $r_0 = 1.6 \times 10^{14} \text{ m} = 0.005 \text{ pc}$). The envelope wind has a mass density of $\rho_* = 3 \times 10^{-17} \text{ kg m}^{-3}$ and a radial velocity of 10 km s^{-1} . The tangential velocity is zero. The temperature is $T^* = 1,000 \text{ K}$; choosing $50 < T^* < 10,000 \text{ K}$ produces similar results. The asphericity is $\alpha = 0.9$, producing an e/p ratio of 10. The steepness parameter $\beta = 6$.

The spherical inner wind has a density $\rho_c = 10^{-20} \text{ kg m}^{-3}$ and a velocity $v_c = 2,500 \text{ km s}^{-1}$. The density contrast between inner and outer winds is then 3,000. The temperature of the fast wind is $T_c = 50,000 \text{ K}$, so the Mach number is $\mathcal{M} = 112$. Four frames of the basic hydrodynamic variables are shown in Fig. 1, at $1.4 \times 10^{10} \text{ s}$ after the onset of the fast wind.

A cool tongue-like deformation extends from the contact discontinuity, forming a chimney along the symmetry axis and helping to collimate the outflow. Turbulent hot gas streams back from the head of the outflow; this exerts a further confining pressure. Finally, the inner 'reverse' shock forms a prolate barrel shape along the symmetry axis, as expected^{3,9}. The fast gas around $\theta \approx 40^\circ$ passes obliquely through this shock, and is thereby deflected upward. The outer layers of the chimney

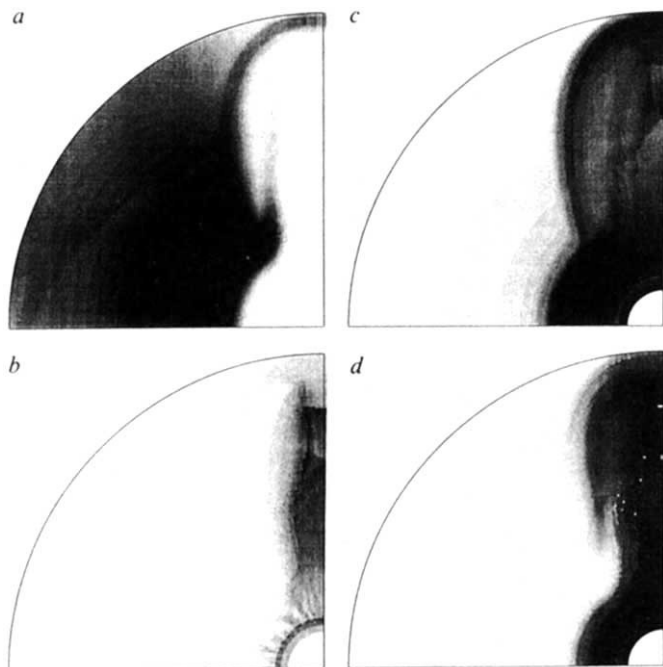


FIG. 1 Equal-histogram⁸ greyscale representation of (a) gas density, (b) absolute velocity, (c) total energy density and (d) temperature. The time is 1.4×10^{10} s (444 yr) after the onset of the fast wind. The radius of each frame is 3.14×10^{15} m (0.1 pc). The equator is at the bottom, and the symmetry axis is at the right edge of each frame. Notice the slightly elliptical inner shock, the almost uniformly hot bubble, and the polar protrusions. The shock structure is most obvious in the plot of the absolute velocity (b). Note especially the contact discontinuity being dragged along, and the turbulent mixing of the cold with the hot gas as a light streak parallel to the symmetry axis where the chimney of cool material is pulled into the hot bubble. X-shaped shocks across the jet can be seen along the axis, and terminal X-shocks near the tips of the outflowing column. Note also the adiabatic cooling of the gas as it expands away from the inner shock, and the abrupt heating (darker shading) of the gas as it passes the X-shocks. The white pixels in d are due to rounding errors in calculation of the temperature. Hot material accumulates on the outside of the sheath of entrained gas, and its pressure contributes to the autocollimating effects of the flow.

therefore move faster than the jet core; this leads to vortex clouds and axial condensations.

The advancing parts of our jets are very similar to known shapes. For example, a pair of X-shaped shocks^{10–12} forms across the symmetry axis. Figure 2 illustrates the complex motions in the flow. The autocollimation is due to refraction of the fast gas across the inner shock, inertial confinement by the chimney and the pressure of the backflow gas outside it. This mechanism is quite different from the Blandford-Rees¹³ nozzle.

The generic features of our simulations are these. (1) The value of β mostly determines the degree of collimation of the flow. (2) The contact discontinuity establishes, for $\beta > 4$, a turbulent chimney. This steeply curves back on itself just inside the outer shock. It is this configuration that produces remarkable jet-like features. For $\beta \approx 3$, the flow near the symmetry axis terminates in shocks of the type seen^{11,12} in cylindrical jets. (3) For $\beta < 3$, the inner shock is strongly aspherical, whereas larger values of β produce more-spherical inner shocks.

We have verified that the collimation mechanism is not sensitive to T_c : it works down to $M \approx 15$. Also, it allows wind density contrasts as low as 1:100. The collimation is purely geometrical; as long as partial waves propagate faster along the axis than in the equatorial plane, the mechanisms of cusp formation (with its consequent chimney) and focusing through the inner shock still operate.

We have run simulations that extend radially three times as far as the cases depicted here, without seeing any signs of decollimation. Limitations of computer time preclude more extended runs, but our simulations produce the perfect instream conditions assumed¹² in cylindrical jet simulations. Such computations therefore start where we stop.

The model parameters can be changed according to the usual hydrodynamic scaling rules. They allow the application of our PN models to other flows with the same basic geometry, such as might occur in active galactic nuclei¹⁴ and in young stellar objects. For definiteness, we consider parameters^{15,16} that are often accepted for active galactic nuclei. A length scale of $\lambda = 3 \times 10^{16}$ m, velocity $v \approx c$ and luminosity $L = 10^{41}$ W yield a timescale of 10^8 s. This is close to the fastest changes noted in the emission lines of H α and H β , but much shorter than the lifetimes of 10^{15} s inferred for giant radio doubles. This long

timescale (about a galactic year) is probably related to the feeding mechanism of the galactic nucleus. The density in a jet of cross-section S can be inferred from $L \approx \rho v^3 S$, and if $S \approx 0.1 \lambda^2$, we get $\rho \approx 4 \times 10^{-17}$ kg m⁻³. If the ambient density is taken as that of the intercloud medium in the broad line region, the (outer/inner) density ratio is 10^3 – 10^4 . The broad line width is $\sim 5,000$ km s⁻¹; this should be roughly equal to the local sound

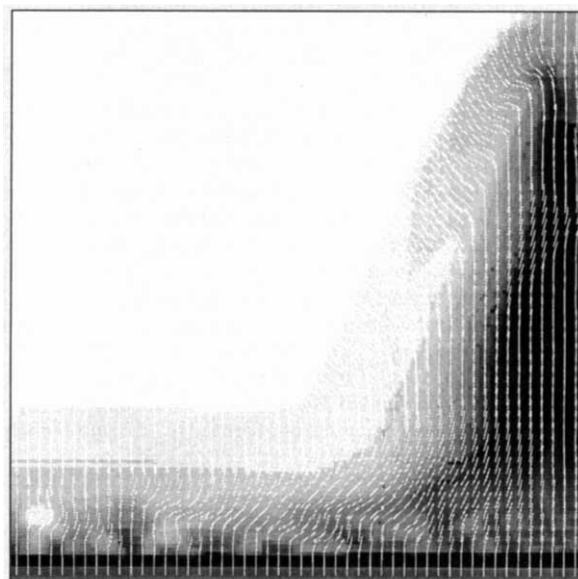


FIG. 2 Equal-histogram greyscale rendering of the absolute value of the velocity, with the direction of the flow superposed in white lines. Ordinate is the radial direction r , abscissa is the polar angle θ decreasing from $\pi/2$ on the left to zero on the right of the panel. Notice the sharp outer shock, and the fact that the contact discontinuity is strongly deformed. Also note the artificial ripples located a few cells beyond the inner shock, which is visible as a black band at the lower edge of the frame. These ripples are due to time splitting errors which occur when a shock crosses a grid zone at a very small angle. The ripple amplitude is usually not more than $\sim 10\%$. Clearly, the gas is preferentially deflected by the inner shock near $\theta \approx 40^\circ$, which helps the collimation.³

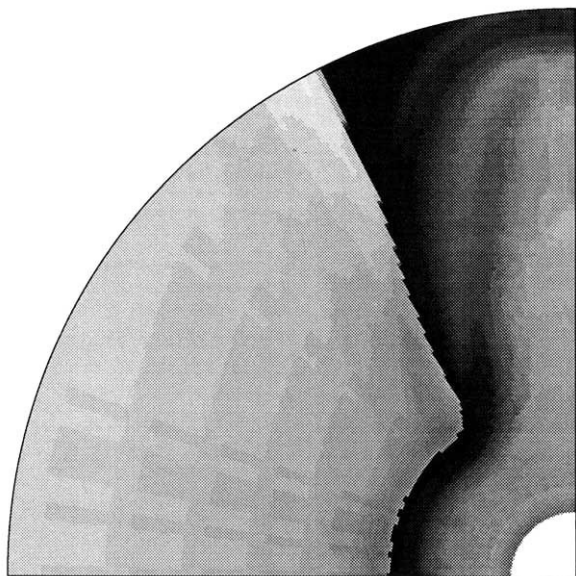


FIG. 3 Emissivity due to all hydrogen recombination lines, calculated in the radial-only approximation¹⁷. Parameters as in Fig. 1, using a Lyman continuum flux of 10^{44} photons s^{-1} .

speed, so that the Mach number at the onset of the fast wind is $M \approx 60$. Our model produces collimated outflow for all these parameter values.

A final interesting effect occurs once we include photoionization¹⁷ by the central object. If the shock-compressed density of the envelope is high enough, it can capture the Strömgren region, which would appear as a barrel-like section around the symmetry axis. If the jet is well developed, the hole it punctures in the envelope lets the Lyman continuum photons escape. This produces a pair of ionization cones (Fig. 3) emanating from the ends of the small barrel. Such features are observed¹⁸ near active galactic nuclei. Thus, the same mechanism that forms jets accounts for the ionization cones.

Our model can simply account for one-sided jets¹⁹ by breaking the reflection symmetry across the equatorial plane of the envelope. This should show up strongly in the shape of the Strömgren region. Thus, if a consistent one-sidedness of ionization cones were observed in active galactic nuclei, it would produce evidence in favour of the 'flip-flop jet' picture^{19,20}.

Extremely well collimated jets can be made by our aspherical interacting-winds model. The jets are collimated within a few envelope length scales at most. The collimation angle is almost immeasurably small if $\beta > 5$, notwithstanding the fact that the opening angle of the confining envelope can be as large as a radian or more. Similar calculations with a relativistic hydro-code²¹ show that relativistic jets are formed by the same mechanism, and in fact become even better collimated. $\square$

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Spatially resolved near-infrared emission and a bubble of hot gas in the central active region of the Galaxy

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It has been known for almost two decades that there is a compact, nonthermal radio source within one arcsecond of the dynamical/gravitational centre of our Galaxy^{1,2}. This source, designated SgrA*, is regarded as the most likely candidate for a central black hole³. Evidence for an active central source has, however, so far been lacking at infrared wavelengths⁴⁻⁸. Here we report near-infrared speckle and imaging spectroscopic observations which resolve the central complex (comprising SgrA* and the infrared source IRS16) into about two dozen compact sources. An expanding bubble of hot gas is found to be centred within 3 arcsec of SgrA*. There is also evidence of a blue object, positionally coincident with SgrA*, emitting at 2 μm . The hot bubble is probably created by shock excitation of a fast wind. These observations are consistent with the presence of an accreting massive black hole at the Galactic Centre.

We report here the results of two recent experiments. First, we used the new MPE near-infrared high-resolution camera, SHARP, at the New Technology Telescope (NTT) of the European Southern Observatory (ESO) at La Silla, Chile, for near-diffraction-limited imaging at H (1.6 μm) and K (2.2 μm) of the IRS16/SgrA* complex. SHARP uses a 256×256 pixel NICMOS III detector array developed by Rockwell International Corporation. A system of digital signal processors allows read-out of the array as fast as 10 Hz, on-line flat-fielding, dead pixel correction, and some on-line data processing (such as shift-and-add⁹). For the Galactic Centre observations on 20–23 August 1991, we used an image scale of 0.05" per pixel and read out one quadrant of the array with integration times of 0.5 and 1 s. The data analysis used shift-and-add⁹ and Knox-Thompson¹⁰ algorithms and is briefly explained in Fig. 1. Second, we observed an unidentified emission line at 2.217(1) μm that was found by Allen *et al.*⁷ to originate within a few arcsecs of IRS16 and SgrA*. The data were taken on 5 August 1991 with the MPE imaging spectrometer FAST⁷ on the 4.2-m William Herschel Telescope (WHT) on La Palma, Spain. The pixel scale of the 62×58 InSb array was 0.5" with seeing producing a resolution of $\sim 1.6''$ (full width at half maximum). The spectral resolution was 300 km s^{-1} . Two sets of images were taken sequentially at velocity offsets 0, ± 300 and $\pm 1,200$ km s^{-1} from the nominal line position (integration time 200 s each) and two sets at offsets 0, ± 300 , ± 600 and $\pm 1,500$ km s^{-1} (integration time 100 s). Following dark-current subtraction, flat-fielding and sky subtraction, the data were divided by the spectral response

of featureless stars in the Galactic Centre. After aligning all bright stellar images, line images were created by subtracting the continuum (an average of the outermost velocity offsets on either side). The wavelength calibration is accurate to $\sim \pm 30 \text{ km s}^{-1}$.

We now discuss the results of the speckle observations. The K-band image (Fig. 1) agrees well with other recent K-band data⁴⁻⁶, but has substantially higher resolution ($0.25''$) and greater point-source sensitivity ($K = 15$, dynamic range $\Delta K \geq 8$). The central IRS16 complex now splits up into about two dozen compact sources. Lunar occultation observations show that the brightest of these (16NE, NW, C, SW-W) must be single stars, as their size is less than a few 10^2 AU (refs 12, 13). There is no significant large-scale extended emission in Fig. 1. The extended ridge in 16SW-E found by other authors^{5,12} is resolved into at least 5 stellar sources. IRS16NE, CC, C, NW, SW and 33 show broad ($\geq 1,000 \text{ km s}^{-1}$ full width at zero power) $2\text{-}\mu\text{m}$ He I emission lines indicative of dense, fast winds from hot stars⁷. We identify these stars with blue supergiants, probably luminous blue variables⁷. Detailed analysis of the characteristics of the He stars will be presented elsewhere (A.K. *et al.*, manuscript in preparation). Furthermore, we detect a faint source ($K = 13.7 \pm 0.6$ or 2 mJy , at $0.25''$ resolution) coincident with SgrA* within the systematic relative positional uncertainty of $\pm 0.3''$ (Fig. 1). From the offset to SgrA* in Fig. 1 and the absolute position of SgrA* (RA = 17 h 42 min 29.316 s, Dec = $-28^\circ 59' 18.38''$ [1950],

ref. 14), we derive an absolute position for the new infrared source of RA = 17 h 42 min 29.31 s, Dec = $-28^\circ 59' 18.5'' (\pm 0.3'')$. It is part of a $1''$ north-south ridge of emission and may itself be spatially extended on a scale of $\sim 0.5''$ with an integrated K magnitude of ~ 12 . The source is also clearly detected on the H-band image that we obtained during the same observing run (integrated $H = 13.9$). After correcting for interstellar dust extinction ($A_K = 3.4$, $A_H = 5.4$, ref. 6) we find it has a blue colour (similar to IRS16NE), consistent with the Rayleigh-Jeans tail of a black body of temperature $T \gg 7,000 \text{ K}$ and a luminosity of $4 \pm 2 \times 10^5 (T/35,000 \text{ K})^3 L_\odot$. An identification of the new source as the near-infrared counterpart of SgrA* can only be tentative at present, as in a region as crowded as the central few arcsecs (about 1.2 sources with $K \leq 13.7$ per arcsec²) the probability of a chance alignment is substantial ($\sim 10\%$). Additional observations (such as proper motions) of the new source are required for unambiguously establishing a physical connection with SgrA*.

We next turn to the interpretation of the $2.217\text{-}\mu\text{m}$ line emission. Our measurements show a moderately intense ($> 10^{-4} \text{ erg s}^{-1} \text{ cm}^{-2} \text{ sr}^{-1}$), spatially extended emission line of observed width $\sim 500 \text{ km s}^{-1}$ (FWHM), consistent with ref. 11. It is centred within a third of a resolution element of the wavelength of the $[\text{Fe XI}] \ ^2\text{D}_{5/2} \rightarrow \ ^2\text{D}_{3/2}$ transition at $2.217(5) \mu\text{m}$, as established from ultraviolet observations of the solar corona in ref. 16. But a more recent evaluation³³ places

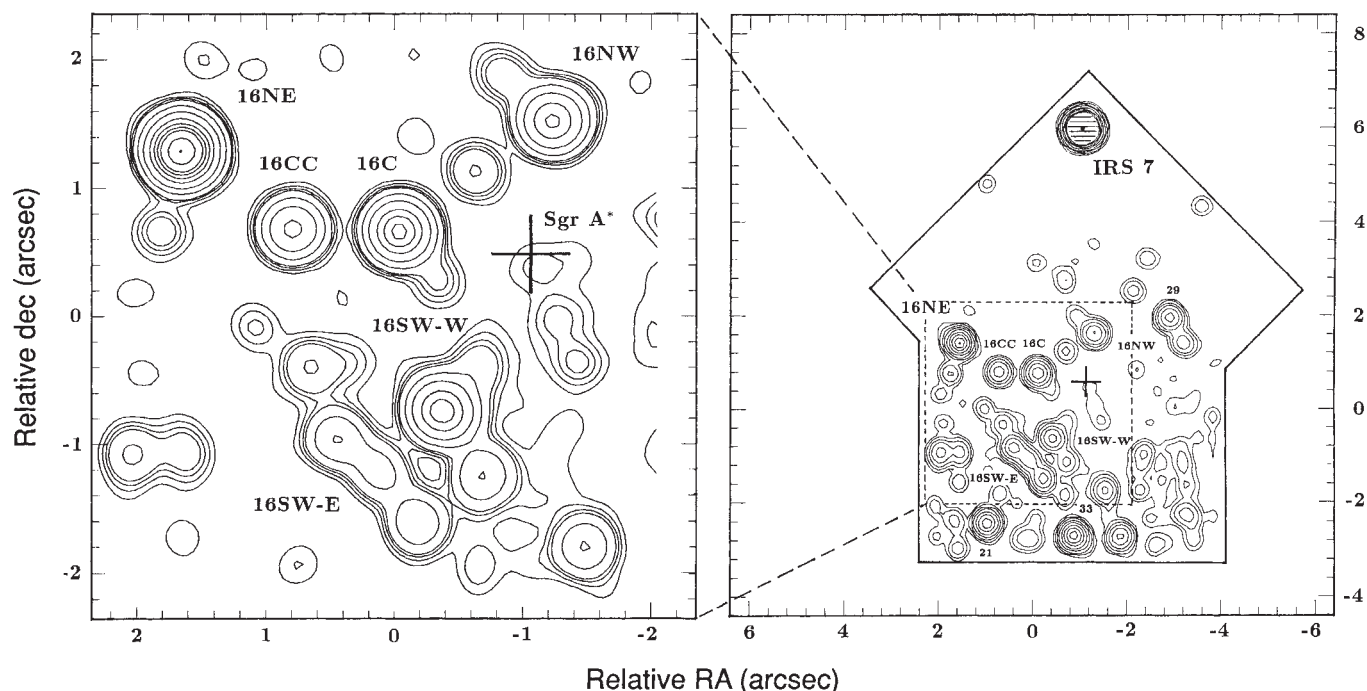


FIG. 1. High-resolution K-band image of the central $7'' \times 10''$ of the Galaxy. Right, a mosaic of two sub-images, at resolution $0.25''$ FWHM, of the entire area observed, with the outer boundaries of the two sub-images indicated. Standard designations of the brighter sources are given and the cross denotes the position of SgrA*. The lower sub-image contains $\sim 2,000$ frames of integration time 0.5 or 1 s, the upper 5,000 frames of 1 s. Contour levels are 1, 2, 4, 8, 16, 30, 50, 75 and 100% of the peak flux of IRS16NE ($K=8.7$, ref. 5). The lower sub-image was processed with a shift-and-add algorithm⁹ with IRS16NE as the reference source. The upper image was reduced with a Knox-Thompson algorithm¹⁰ to increase the dynamic range in the presence of the bright ($K=6.8$) reference source IRS7. Residual dirty beam structures apparent at a few % of the reference sources were then removed iteratively by a Lucy deconvolution³¹. After weighted adding, the two sub-images were then smoothed with a gaussian back to $0.25''$, the resolution of the shift-and-add/Knox-Thompson process. Left, the deepest part of the image in the

overlap area of the two sub-images. The data are smoothed here to $0.3''$ resolution to further increase sensitivity ($K=15.5$). Contour levels are 1, 1.5, 2, 2.5, 5, 10, 20, 30, 40, 50, 75 and 100% of $K=8.7$. To register SgrA* on the map, three techniques were combined. First, we used the best absolute radio and infrared positions in the literature, obtaining absolute positions for our K-band sources from the observed offsets from IRS16NE and IRS7 and the absolute positions in ref. 14. Second, we used offsets of SgrA* from IRS7 derived from recent radio maps that show both sources^{23,28}. Third, we derived relative positions from a comparison of Br α and radio continuum maps of the ionized gas³². The position of SgrA* and its uncertainty are derived from an average of these methods and agree well with positioning and systematic error bars obtained previously^{4,5,14,32}. A new $K=13.7 \pm 0.6$ source is located $0.18''$ west and $5.5''$ south of IRS7 ($\pm 0.1''$), within $0.2''$ of the nominal position of SgrA*.

the [Fe XII] line at $2.205(1) \mu\text{m}$. There is also a transition of [Fe III] (${}^3\text{G}_5 \rightarrow {}^3\text{H}_6$) at $2.2178(20) \mu\text{m}$ (ref. 34). No other well known atomic or molecular emission lines observed in standard H II regions lie close to this wavelength. An identification of the newly discovered emission line with either the [Fe XII] or [Fe III] transition is thus very likely, although the exact velocity of the line emission remains uncertain until a better determination of the rest wavelength becomes available. Both lines trace hot material; Fe XII has an excitation potential of 290 eV and Fe III of 16 eV. The distribution of the line emission at nominal velocity of 0 km s^{-1} relative to the local standard of rest (v_{LSR}) is shown in Fig. 2 superposed on a radiograph of the 15-GHz radio continuum¹⁵. It forms an arc of $2''$ radius which seems to surround a 'mini-cavity' in the radio continuum 'mini-spiral' pointed out in refs 15, 17 (Fig. 2). The line emission at $v_{\text{LSR}} = +300$ and -300 km s^{-1} is centred inside the cavity and within $\sim 3''$ of SgrA*. This suggests that the mini-cavity contains a bubble of hot gas and is expanding at a few hundred kilometres per second.

It can be excluded that the hot bubble is photoionized. The spatial distribution of the $2.217\text{-}\mu\text{m}$ line is very different from the photoionized gas in the SgrA West H II region, and in the case of Fe XII, the relatively soft ultraviolet spectrum in the Galactic Centre ($T_{\text{eff}} \leq 35,000 \text{ K}$, ref. 7) cannot provide the number of photons of energy $\geq 290 \text{ eV}$ required to explain the velocity-integrated peak line intensity ($4 \times 10^{-4} \text{ erg s}^{-1} \text{ cm}^{-1} \text{ sr}^{-1}$). This implies that the hot bubble is collisionally ionized. Calculations for Fe XII show that its fractional abundance is maximum at temperatures from 1×10^6 to $3 \times 10^6 \text{ K}$ ($\text{Fe XII}/\text{Fe} \approx 0.25$) and decreases rapidly outside that range¹⁸. Within this temperature range and for a critical density of collisional excitation of the ${}^2\text{D}_{5/2}$ state of $6 \times 10^8 \text{ cm}^{-3}$ (M. Greenhouse, personal communication) the Fe XII bubble requires an emission measure of gas at 10^6 K of $n_e N_e = 2 \times 10^{25} \text{ cm}^{-5}$ and a total mass of $0.1 \times (4 \times 10^4/n_e) M_\odot$. Calculations of the line and continuum emission from a hot plasma¹⁹ show that the Fe XII bubble must emit $10^5 L_\odot$ or more of soft ($\sim 100\text{-eV}$) X-rays. This X-ray radiation may be a significant fraction of the observed $\sim 1\text{-keV}$ flux observed by the Einstein satellite²⁰ and also contributes to the ionizing luminosity of the central parsec. If the line emission is due to [Fe III], the gas temperature must be between 4×10^4 and $1 \times 10^5 \text{ K}$. The amount of gas may then be similar to what was estimated for Fe XII, but the contribution to X-ray flux and ionization would be greatly reduced.

As an explanation of the hot bubble, we propose that a fast wind originating within $2''$ of SgrA* expands into the streaming gas ($v \approx 250 \text{ km s}^{-1}$, ref. 21) of the radio mini-spiral, in agreement with earlier ideas for the mini-cavity^{15,17}. As the mini-cavity is closed toward the east and west, we find it implausible that this wind comes from the He I stars in IRS13 or the IRS16 complex. In our opinion our new observations, taken together with the recent detection of an ionized gas tail stretching northward from IRS7, roughly away from SgrA* (refs 22, 23), naturally suggest that SgrA* itself may be the source of a strong wind. The alternative possibility that the mini-cavity and [Fe XII] emission have been created by a recent supernova explosion is less likely. The hydrogen mass surrounding the mini-cavity is less than $0.5 M_\odot$ so that the supernova would still be in its free expansion phase. The expansion velocity of the hot bubble is much less than the several 10^3 km s^{-1} expected for the supernova ejecta and swept-up shell during that phase. In addition the radio emission in and surrounding the mini-cavity is thermal, unlike in supernova remnants.

In the wind model it is possible to apply spherically symmetric wind bubble theory²⁴ for a first-order analysis of the wind parameters, independent of line identification. In this model the $2.217\text{-}\mu\text{m}$ line is shocked wind or streamer material, whereas the radio continuum and H I Br α emission around the mini-cavity comes from swept-up, shocked gas in the mini-spiral. As input parameters we use the observed size (0.2 pc), expansion

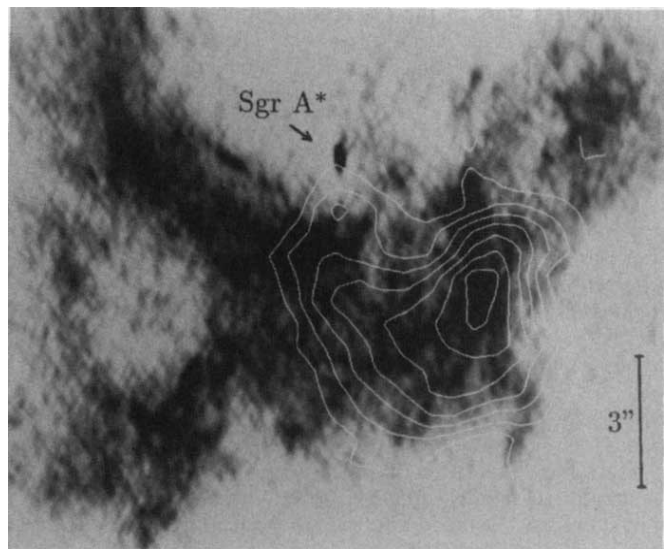


FIG. 2 Contours of $2.217\text{-}\mu\text{m}$ line emission at $v_{\text{LSR}} = 0 \text{ km s}^{-1}$ (rest wavelength $2.217 \mu\text{m}$) superposed on radiograph of 15-GHz radio continuum emission (resolution $0.2'' \times 0.1''$) of ref. 15. The scale is $15.8'' \times 12.8''$. The resolution of the near-infrared data is $1.6''$ FWHM and the contour unit is $5.7 \times 10^{-5} \text{ erg s}^{-1} \text{ cm}^{-2} \text{ sr}^{-1}$. SgrA* is marked by an arrow. The 'mini-cavity' discussed in the text is the U-shaped depression of radio emission centred $3''$ south-west of SgrA*.

velocity (100 to 300 km s^{-1}), mean electron density ($5\text{--}10 \times 10^3 \text{ cm}^{-3}$), peak electron density ($\sim 10^5 \text{ cm}^{-3}$) and column density in the mini-spiral surrounding the mini-cavity ($N_e = 3 \times 10^{21} \text{ cm}^{-2}$). These parameters are all reasonably fitted by a wind of mechanical luminosity $0.5 \dot{M}_w v_w^2 = 10^{38.7 \pm 0.5} \text{ erg s}^{-1}$ and an age of 10^3 years. The inferred bubble age is an excellent agreement with the time that the streaming gas spends in the vicinity (0.2 pc) of SgrA*/IRS16. If the tail of IRS7 is struck by the same wind that creates the mini-cavity, another constraint can be derived. The velocity field and shape of IRS7's tail in the $12.8\text{-}\mu\text{m}$ [Ne III] line indicate that the ratio of wind force to total gravitational force is ~ 0.3 , or $\dot{M}_w v_w \approx 3 \times 10^{30} \text{ dynes}$ (ref. 22). If we allow for a significant contribution to this wind pressure by the He I stars of the IRS16 cluster⁷, the estimates of wind luminosity and pressure can be combined to yield a lower limit to the wind velocity of 10^3 km s^{-1} . For Fe XII, a third constraint can be derived from theoretical models of fast shocks²⁵. The column density of $1\text{--}3 \times 10^6 \text{ K}$ gas behind a shock of velocity $v_s = v_w/(1,000 \text{ km s}^{-1})$ is $N_{\text{hot}} \approx 10^{20} v_s^{-1}$ (ref. 25). Combined with the observed emission measure and folded with the bubble geometry with several shock fronts along any line of sight, this implies an electron density in the hot gas of a few 10^4 cm^{-3} and a total wind mass loss rate of $\sim 10^{-2} M_\odot \text{ yr}^{-1}$. To be consistent with the wind luminosity estimated above, the wind velocity cannot be much greater than 10^3 km s^{-1} . The presence of a hot wind bubble may also help to explain the lack of radio recombination lines toward this region^{26,27}, either through collisional ionization of high Rydberg states or through optical depth effects in the radio continuum emission from the thin, dense swept-up shell.

What does this tell us about the nature of SgrA*? Both the near-infrared source positionally coincident with the compact radio source, and the hot gas bubble in its immediate vicinity, may be physically related to SgrA*. Previous discussions² have emphasized the similarity of SgrA*'s radio spectrum to the optically thick synchrotron emission in relativistic extragalactic jets, albeit at a much lower level of activity than in those luminous sources. The compactness of the mini-cavity in relation to the overall size of the mini-spiral in the vicinity of SgrA*

($\geq 10''$), together with three compact radio blobs that seem to be connecting SgrA* to the mini-cavity^{28,29} roughly along the extended ridge of 2- μ m emission south-west of SgrA* (Fig. 1), may indicate that the wind is anisotropic. The wind luminosity we have estimated from the characteristics of the mini-cavity is almost two orders of magnitude greater than the wind luminosity of even a very massive ($100M_{\odot}$) star (10^{37} erg s⁻¹, ref. 30). If this wind is associated with SgrA* as we propose, SgrA* cannot be a normal star. The inferred parameters of SgrA* ($L \approx 0.5\dot{M}_w v_w^2 \geq 10^5 L_{\odot}$) are, however, plausible for low-efficiency accretion and subsequent ejection of matter from an accretion disk around a $10^6 M_{\odot}$ black hole. $\square$

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Superconductivity at 8.4 K in calcium-doped C₆₀

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IT has been demonstrated^{1–6} that solid C₆₀ can be readily intercalated with group IA alkali metals to give metallic or insulating compounds, depending on the dopant concentration. The metal atoms diffuse into tetrahedral and octahedral interstitial sites of the C₆₀ lattice with little disturbance to the face-centred cubic (f.c.c.) packing⁷. The A₃C₆₀ f.c.c. phases (A is K, Rb, Cs and mixtures of these) exhibit superconductivity with a transition temperature that increases with lattice constant⁸. At higher dopant concentration a body-centred tetragonal A₄C₆₀ phase⁹ and an insulating, body-centred cubic A₆C₆₀ phase¹⁰ are found. Here we report that the divalent group IIA intercalant calcium can be intercalated into the f.c.c. sites of C₆₀ to form a solid solution, and that, near a Ca:C₆₀ ratio of 5:1, a phase transformation occurs to a simple cubic phase. Measurements of microwave loss, magnetic susceptibility and Meissner effect show that the simple cubic phase becomes superconducting below 8.4 K.

Fullerenes were synthesized in a 150-kW arc-furnace under conditions similar to those in ref. 11, with a soot yield of ~ 10 g h⁻¹. Fullerenes, extracted with toluene, constituted ~ 5 wt% of the soot and contained $\sim 93\%$ C₆₀. C₆₀ was separated by liquid chromatography and vacuum-outgassed at 200 °C to remove solvents. All sample preparation was done in a controlled-atmosphere glove box where the oxygen or water vapour concentrations are maintained below a few parts per million. High-purity (99.99%) dendritically solidified calcium metal is broken into powder, weighed and mixed with C₆₀ in high-purity tantalum cells. The same cells are used to press the powder mixtures to make pellets. Samples of Ca_xC₆₀, with $x = 1.5, 3, 4, 4.5, 5, 6$ and 8 , were prepared and loaded into quartz tubes without removing them from the tantalum cells. Earlier experiments used quartz tubes alone, and unpressed powder mixtures. Although results for these samples were mostly reproducible, quartz reacts with calcium, and tantalum cells were needed to

avoid sample contamination by unwanted reaction products. The quartz tubes are then connected to ultra-high-vacuum valves, taken out of the dry box and sealed under a vacuum of 10^{-6} Torr. Heat treatments were done in tube furnaces at 550 °C for 20 h. We find that the reaction is nearly complete after only 1 h at this temperature. Following heat treatment, samples were removed from the tantalum cells in the glove box, loaded into 1-mm quartz X-ray capillaries, and again sealed under high vacuum. In another set of experiments to determine the appropriate reaction temperatures, weighed and mixed powders were sealed in X-ray capillaries and heated in an *in situ* X-ray furnace while we probed the structural changes by X-ray diffraction.

Magnetic-field-dependent microwave absorption was used to screen samples for superconductivity. Superconductivity was first detected at 8.4 K in uniformly mixed samples heat-treated at 600 °C for 15 h. The superconducting portions of these early samples were estimated to be in the microgram range, and electron spin resonance g-values indicated that C₆₀ was decomposing in a competing process. Resonances were observed at

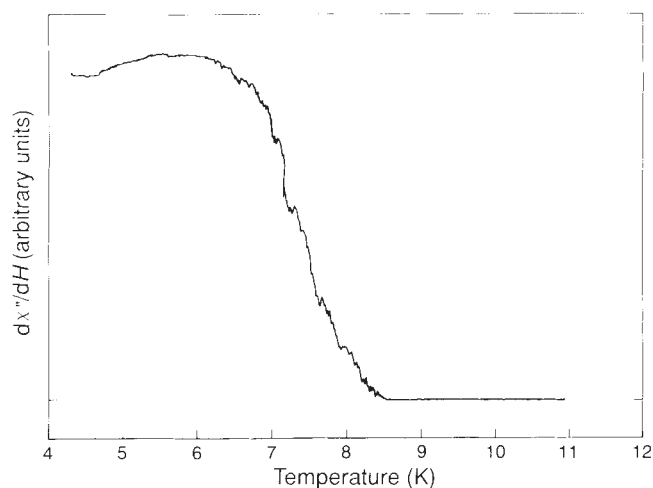


FIG. 1 Microwave loss measurements for Ca₅C₆₀ in a static field of 100 Oe.

$g = 2.0024$ and 1.9995 . The former is characteristic of carbonized material and is attributed to thermal decomposition, and was found in all samples, superconducting or not. The magnitude of this signal decreased considerably for samples heat-treated at 550°C . The lower g -value has been reported for many C_{60} radical salts^{12,13}. Its linewidth is strongly temperature-dependent, ~ 70 G at 300 K and 3 G at 4 K. Integrated intensities revealed Curie behaviour ($1/T$) between 110 K and T_c . Independent of the superconducting fraction, a transition temperature of 8.4 K was observed for all superconducting samples, indicating that the superconducting phase is a line compound, similar to that seen for A_xC_{60} . Figure 1 shows the microwave absorption measurement for the $x = 5$ sample, with an onset at 8.4 K in a field of 100 Oe at 9 GHz.

A SQUID magnetometer was used to measure the temperature dependence of the d.c. magnetization of the samples. Figure 2 shows these measurements for the same $x = 5$ sample as in Fig. 1. The sample is first cooled to 4.6 K in zero field (ZFC) and then gradually heated in an applied magnetic field of 5 Oe. This applied field is excluded to 8.2 K. At $T = 4.6$ K, a diamagnetic state with 14% shielding is measured. This value should be seen as a lower bound on the shielding fraction, because of the strong finite-size effect in granular superconductors. Magnetic flux expulsion during the superconducting transition (the Meissner effect) is observed when the sample is cooled in a field of 2 Oe. These measurements find a Meissner fraction of 5.4%. Some of our samples had as much as 45% ZFC volume fraction and 10% Meissner fraction.

X-ray diffraction measurements were done with Mo $K\alpha$ radiation on a 12-kW rotating-anode generator equipped with a triple-axis goniometer. Highly oriented ZYA graphite crystals were used to monochromatize and analyse the X-ray beam with a longitudinal resolution of 10^{-2} Å. Samples were rocked 6° at each 2θ data point to obtain a statistically averaged powder pattern. Figure 3 shows powder diffraction patterns for six different compositions. Peak positions in these patterns are indexed to a f.c.c. pattern, and good agreement is found with the calculated positions. The same fitting routine yielded the lattice constants for each composition. In the $x = 1.5$ pattern, there are no diffraction peaks that can be attributed to a non-f.c.c. unit cell or to calcium metal. As the calcium composition increases, peak intensities change but no new peaks appear for $x < 5$. This suggests that, in this range of compositions, calcium- C_{60} is behaving like a solid solution, with calcium atoms populat-

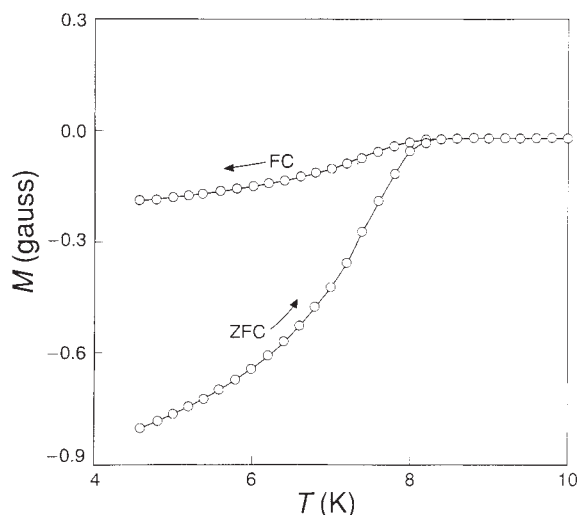


FIG. 2 Magnetic susceptibility measurements of Ca_5C_{60} sample, demonstrating diamagnetism in zero-field-cooled and field-cooled cases.

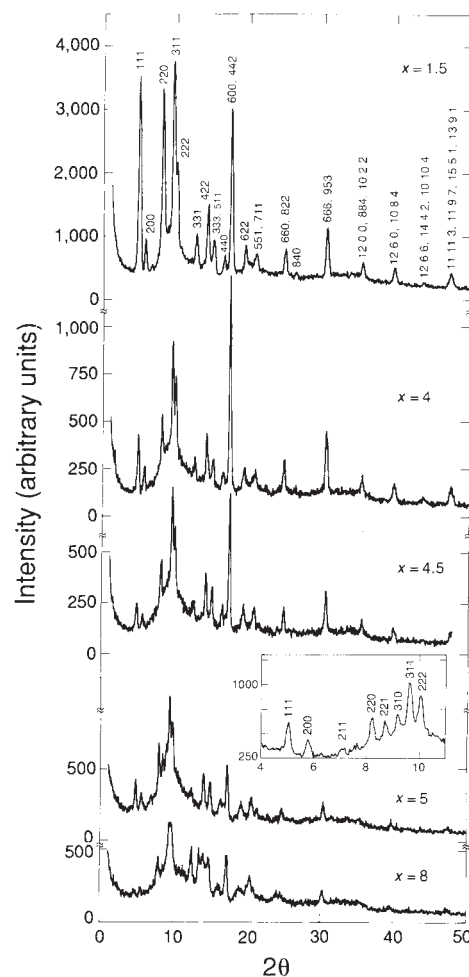


FIG. 3 Powder X-ray diffraction patterns of Ca_xC_{60} reveal structural changes with increasing calcium concentration. Inset: magnified region where the simple cubic reflections are observed for $x = 5$.

ing the tetrahedral and octahedral interstitial sites without significantly disturbing the host f.c.c. lattice.

In the pristine f.c.c. C_{60} structure, there are one octahedral and two tetrahedral sites per C_{60} molecule. If singly filled, in principle three is the maximum number of intercalant atoms per C_{60} the interstitial sites can accommodate. These vacant sites have a radius of 1.12 Å for the tetrahedral and a centred radius of ~ 2.07 Å for the octahedral site. On steric grounds alone it may seem that up to eight 0.99 -Å radius Ca^{2+} atoms can be accommodated in this vacancy, one on each of the eight three-fold corners of the octahedral site. This will place calcium ions 2.8 Å apart, at these threefold corners. (For Ca^{2+} these sites are calculated as $(0.40, 0.40, 0.40)$ and its symmetry-equivalent positions.) Such a separation for calcium ions may not be allowed on chemical grounds. A more plausible occupancy may be four, where four calciums are spaced 4.0 Å apart at the diagonally opposite sites. Such a decoration would increase the number of intercalant atoms from three to six per C_{60} , without significantly disturbing the f.c.c. cell. Multiply occupied octahedral sites would explain the solid-solution behaviour found for calcium over a broad range of compositions. In fact, peak intensities qualitatively agree with preliminary model calculations carried out using randomly oriented C_{60} molecules in a $Fm\bar{3}m$ symmetry and a gradual filling of the octahedral sites with increasing numbers of calcium ions.

For the $x=5$ sample, we observe strong 211, 221 and 310 peaks, which belong to a simple cubic lattice and are forbidden for a f.c.c. structure, as shown in Fig. 3 (inset). At this composition, where superconductivity is also observed, the system transforms into a simple cubic structure while maintaining the f.c.c. unit cell dimensions. This can be viewed as a loss of symmetry, as the atoms within the unit cell move into symmetrically inequivalent positions. A similar transition was reported¹⁴ for pristine C_{60} , where the orientational ordering of the molecules gave rise to simple cubic lattice peaks at large angles. Comparison with this earlier report, particularly the observation of forbidden peaks at low scattering angles suggests that the phase transition is not due to C_{60} ordering but rather to rearrangement of calcium atoms. This simple cubic structure persists to $x=8$ but becomes less intense, suggesting that the volume fraction of this phase gradually decreases.

The composition dependence of the diamagnetism in zero-field cooling, the lattice constant and an unusual intensity ratio are shown in Fig. 4. Magnetic susceptibility measurements indicate that the diamagnetism peaks strongly near $x=5$ (Fig. 4a). Microwave loss measurements also confirm a maximum superconductivity response near $x=5$. Lattice constants determined from fits to all peaks in the powder pattern decrease sharply in the dilute regime, $x < 1.5$ (Fig. 4b). Near $x=1.5$, the decrease reaches a limiting value, indicating that even at this composition, with only half the interstitial sites filled, calcium atoms are uniformly distributed in the interstitial sites. At this composition, calcium atoms could donate up to three electrons per C_{60} , which would half fill the t_{1u} -derived band, creating a similar situation to that observed in the superconducting A_3C_{60} alkali metal compounds. For calcium, however, we do not detect supercon-

ductivity at this composition. Further increasing the calcium concentration, we observe a second, smaller decrease in the lattice constant near $x=4$, and a sudden increase at $x=5$, which correlates with the phase transition to the simple cubic superconducting phase. At this critical concentration, the unit cell is forced to expand at the expense of losing its f.c.c. symmetry. Increasing x beyond 5 causes an continual expansion in the unit cell, suggesting that highly populated octahedral sites are spreading the molecules apart.

One important point that came to our attention while fitting the structure was a largely underestimated 442,600 peak intensity, especially in the $x < 5$ range. This peak depends strongly on composition, as can be seen in Fig. 3. If this dependence is normalized to the slowly varying 422 reflection, it shows a maximum near $x=3$ (Fig. 4c). This may imply a vacancy ordering. In the dilute regime, calcium atoms fill the tetrahedral and octahedral sites randomly as in a lattice gas system, contributing single Ca^{2+} ions to these sites. Preliminary fits show no preference for one site over the other. Near $x=3$, assuming all tetrahedral and octahedral sites are filled with one calcium, the calcium atoms in the octahedral sites have considerable extra space available to them. It is reasonable that the small calcium ions prefer moving off-centre toward triply coordinated sites along the $[111]$ axis which are also consistent with the $Fm\bar{3}m$ symmetry. When this off-centre occupation becomes non-random, the correlation will have a strong effect on the diffraction peaks. A cooperative shift of the calcium atoms to (0.45, 0.45, 0.45) positions from the usual (0.5, 0.5, 0.5) octahedral site would lead to vacancy ordering, and place extra atoms on the 442 planes, possibly explaining the extra intensity observed. One should then expect the maximum in this intensity to occur near $x=3$, as placing more than one atom in the octahedral sites would force atoms out of the 442 atomic planes. For compositions $x < 3$, when octahedral sites are singly occupied, atomic size considerations alone would allow a larger metallic calcium with 2 Å radius to fit at the centre of this site. If the vacancy ordering argument is correct in this dilute regime, however, the off-centre octahedral position cannot possibly accommodate a metallic calcium atom.

In the superconducting f.c.c. A_3C_{60} alkali metal compounds, each dopant donates one electron to a C_{60} molecule, as the conduction band in C_{60} arises from weak hybridization between the triply degenerate t_{1u} molecular orbitals, the band is half-filled at the composition A_3C_{60} . The ionic radius of the alkali metal cations determines the lattice constant, and this in turn correlates with the superconducting transition temperature through changes in the density of states at the Fermi level^{8,15,16}. It is of some interest that this correlation is continued by the present results (Ca_5C_{60} , $a_0 = 14.01$ Å, $T_c = 8.4$ K). In the case of calcium the degree of charge transfer is less certain, and it is possible that the superconducting Ca_5C_{60} composition also corresponds to an approximate valence of C_{60}^{3-} , especially if calcium clusters are formed in the octahedral sites. Alternatively, if the degree of charge transfer from calcium is high, then the C_{60} t_{1g} -derived band may become populated^{15,16}. Although the correlation of transition temperatures between alkali and alkaline earth C_{60} superconductors would then involve electrons in different bands, the Fermi-level densities of states in the two bands are rather similar. Furthermore, the direct products $t_{1u} \times t_{1u}$ and $t_{1g} \times t_{1g}$ give rise to identical representations, and thus the same C_{60} intramolecular vibrations^{17,18} could supply the electron-phonon coupling for superconductivity in both series of compounds. Moreover, calculations (M. A. Schluter, personal communication) such as those reported in ref. 16 show the coupling strength to be the same, within 10%, for t_{1u} - or t_{1g} -derived states. A phonon-mediated mechanism for superconductivity in alkali-metal intercalated C_{60} is implied by recent isotope substitution experiments^{19,20}.

The observed superconductivity in the calcium- C_{60} compound demonstrates that superconducting C_{60} compounds are

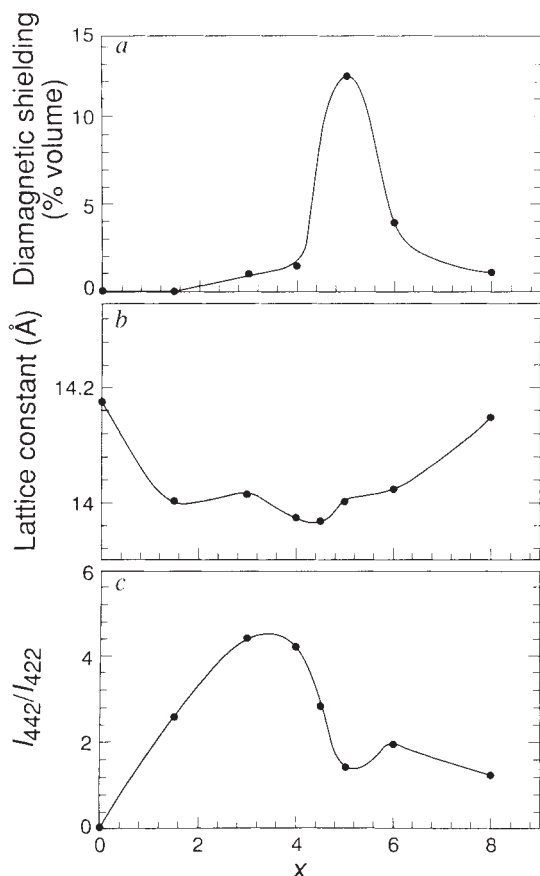


FIG. 4 Correlation of diamagnetism, lattice constant and vacancy ordering with calcium concentrations (see text).

not limited to column IA alkali metals alone, and other systems may exist. While this work was in progress, we became aware of photoemission studies²¹, showing high levels of C₆₀ doping by magnesium, barium and strontium. □

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Infrared reflectivity measurements of a superconducting energy scale in Rb₃C₆₀

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AN understanding of the superconductivity and other electronic properties of the alkali-metal-doped fullerenes¹ will require measurements of their solid-state physical properties. In the normal state one would like to know the electron effective mass, the Fermi energy and conduction bandwidth, and the mean free path for electron transport; the superconducting state, meanwhile, can be characterized at the most basic level by length and energy scales such as the coherence length, penetration depth and superconducting energy gap. Here we describe the use of far-infrared reflectivity measurements to probe the low-energy response of Rb₃C₆₀. We derive a value for the characteristic energy scale associated with reflectivity, which allows us to estimate a value for the gap energy 2Δ of $3\text{--}5kT_c$, in reasonable agreement with tunnelling measurements². Combining this result with data obtained in previous studies, we estimate other length and energy scales, such as the bandwidth and penetration depth.

Far-infrared spectroscopy of superconductors began with work on the transmission characteristics of thin elemental films³. Shortly thereafter Richards and Tinkham⁴ used reflectivity measurements to study superconducting properties of bulk elemental solids. To obtain a characteristic energy scale for the superconducting state, these authors used ratios of superconducting to normal state spectra (examples are shown in Fig. 1a). From these ratios, superconducting energy gaps of

$\sim 3\text{--}4.5kT_c$ were inferred for several elemental superconductors. The agreement (within an experimental accuracy of $\sim 15\%$) between these infrared results⁴ and more precise tunnelling measurements⁵ enhanced confidence in both techniques. More recently, far-infrared reflectivity⁶ (Fig. 1b) has been used to infer an energy gap of $2\Delta/kT_c \approx 3.5 \pm 0.5$ for Ba_{0.6}K_{0.4}BiO₃ ($T_c \approx 30$ K), which has been confirmed by tunnelling data⁷.

The alkali-doped fullerene studied here presents special problems because the superconducting properties are destroyed by exposure to air. For our measurements we sandwich the superconducting material between two sapphire discs, which are sealed around the edge with epoxy. Below ~ 100 K the sapphire becomes transparent for frequencies $\omega \lesssim 300\text{ cm}^{-1}$, and one can measure the internal reflection at the sapphire/Rb₃C₆₀ interface. With these samples the morphology of the sapphire/Rb₃C₆₀ interface (the Rb₃C₆₀ is pressed between the sapphire plates) is certainly less than ideal, but as we are interested in the frequency range from ~ 100 to 10 cm^{-1} , which corresponds to wavelengths of $30\text{--}300\text{ }\mu\text{m}$ (in the sapphire), these surfaces should be acceptable. To study infrared properties at much shorter wavelengths would presumably require smoother surfaces. Measurements were made with a fast scanning interferometer with mylar beam-splitters and a 1.4-K bolometer detector.

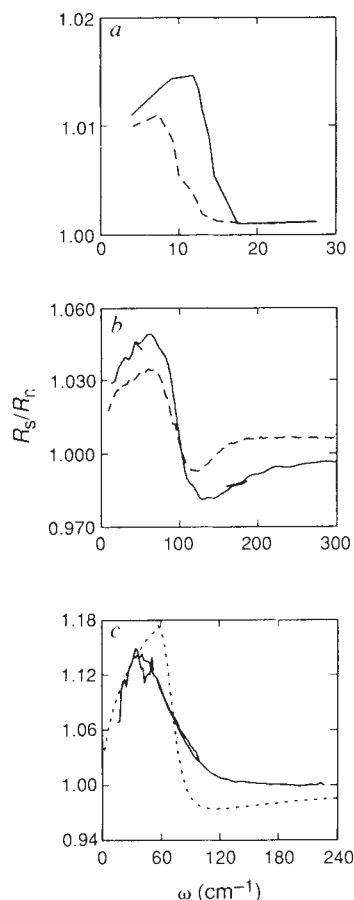


FIG. 1 *a*, The ratio of reflectivity in the superconducting state to that in the normal state measured in a cavity geometry is shown for vanadium (solid line) and tin (dashed). From these data, Richards and Tinkham⁴ inferred energy gaps of $2\Delta \approx 3.5kT_c$ for these superconductors. *b*, Similar reflectivity ratio from Ba_{0.6}K_{0.4}BiO₃, from which a gap $2\Delta \approx 3.5kT_c$ was inferred⁶. The dashed curve is R_s/R_n for YBa₂Cu₃O₇ scaled on the frequency axis by 0.15 (ref. 12). *c*, Ratio of the superconducting (8 K) to normal (35 K) reflectivities for Rb₃C₆₀. The dotted curve shows for comparison a calculation¹⁶ of R_s/R_n for a superconductor/sapphire interface for a moderately dirty superconductor ($\rho_{ac} \approx 0.4\text{ m}\Omega\text{ cm}$) with $2\Delta \approx 60\text{ cm}^{-1}$.

The Rb_3C_{60} powder⁸⁻¹¹ used here was taken from a large batch prepared by the method described in ref. 9. Its quality was checked by X-ray diffraction and magnetometry. The diffraction peak positions and relative intensities are characteristic of essentially phase-pure Rb_3C_{60} (face-centered cubic, $a = 1.442$ nm). A conservative estimate of the crystallite size from the diffraction widths is ≥ 50 nm. A 4-mg sample of the same material, handled in the same glove box, had a zero-field-cooled shielding fraction of 60%, whereas the field-cooled (Meissner) fraction was 8% (ref. 9). These values are comparable to or better than the best reported values. It is believed that the Meissner fraction and transition width are limited primarily by granularity (A. F. Hebard, personal communication) and that the material is more than 80% superconducting, as discussed in ref. 9.

In Fig. 1c we show the ratio, R_s/R_n , of the reflectivity measured at 8 K to that at 35 K. In this ratio one observes a low-frequency enhancement, which is similar to that seen in other superconductors^{4,12} (Fig. 1a and b). The temperature dependence of the reflectivity is shown in detail in Fig. 2a, where we show spectra at 8, 12, 18, 24 and 38 K, each divided by a reference spectrum at 35 K. In Fig. 2b we show the amplitude of the reflectivity ratio at 50 cm^{-1} as a function of temperature. Reducing the temperature from 25 to 12 K causes an increase of $\sim 13\%$ in this reflectivity, whereas a temperature excursion of the same magnitude in the normal state (from 25 to 38 K) leads to a change of less than 1%. This observation explicitly links the low-frequency enhancement of the reflectivity with the occurrence of superconductivity in Rb_3C_{60} .

The issue of measuring polycrystalline samples should also be addressed. For cuprates, a low-energy plasma edge¹³ associated with the almost insulating c -axis response¹⁴ obscured the measurement of the energy gap in polycrystalline samples. In contrast, for the cubic bismuthates ($\text{BaPb}_{0.8}\text{Bi}_{0.2}\text{O}_3$ and $\text{Ba}_{0.6}\text{K}_{0.4}\text{BiO}_3$), infrared measurements of the energy gap with polycrystalline samples are reliable^{6,15}. The difference is that in one case there is an extreme (two-dimensional) anisotropy, whereas in the other there is not. In this regard the doped C_{60} superconductors should resemble the (cubic) BaBiO_3 -derived superconductors, and the fact that measurements are made on non-single-crystal samples should not be a problem.

To estimate the characteristic energy implied by these data, one can compare the data with measurements of other superconductors (Fig. 1a, b), or with calculated reflectivity ratios which show the expected behaviour for ordinary superconductors. A typical ratio calculated by the method of Bickers *et al.*¹⁶ (with an energy gap of 60 cm^{-1} and a normal state conductivity of $2,500\text{ }\Omega^{-1}\text{ cm}^{-1}$) is shown by the dotted curve. Although the calculated ratio drops more rapidly than the measure one, it is clear that the characteristic energy scale associated with the reflectivity enhancement lies between $\sim 45\text{ cm}^{-1}$, where the peak occurs, and 85 cm^{-1} , where R_s/R_n is close to one. Slightly more involved calculations suggest that the data may reflect a distribution of gaps (as found in Al5 compounds¹⁷), from ~ 45 to 85 cm^{-1} , or the presence of a modest fraction of normal material ($\leq 30\%$). The absence of a region in the data where $R_s/R_n < 1$ may be associated with the unusual morphology of the interface. The transition temperature implied by the temperature dependence of the infrared data is slightly lower than that inferred from the susceptibility curve, introducing some uncertainty in our estimate of T_c . We estimate from these data an energy scale of roughly $65 \pm 20\text{ cm}^{-1}$, which if interpreted as a gap corresponds to $2\Delta/kT_c \approx 3-5$. Our upper limit is in reasonable agreement with the value reported by Zhang *et al.*² on the basis of point-contact tunnelling.

One can use this characteristic energy, together with the reported length scale¹⁸ of $\xi \approx 2$ nm, to obtain the characteristic velocity of a carrier near the Fermi level in Rb_3C_{60} . The relationship between the Fermi velocity, v_F , and the superconducting coherence length and energy gap was originally derived by

Pippard¹⁹, and is based on the momentum-position uncertainty principle²⁰. It can be roughly expressed as the inequality, $v_F \leq \xi_0 \pi \Delta / \hbar$. Allowing for a modest difference between the measured coherence length, $\xi \approx 2$ nm, and the coherence length in the absence of disorder²⁰, ξ_0 , we estimate $\xi_0 \leq 3$ nm. Together with an upper bound of $2\Delta \leq 85\text{ cm}^{-1}$ from our infrared measurements, this leads to $v_F \leq 8 \times 10^6\text{ cm s}^{-1}$. This is an unusually low velocity (in conventional metals $v_F \approx 10^8\text{ cm s}^{-1}$), and implies very low electronic response in Rb_3C_{60} .

From formulae for independent electrons in a spherically symmetric band²¹, the above Fermi velocity would imply a Fermi energy of $E_F \leq 100$ meV. Although neither of these approximations is strictly appropriate for Rb_3C_{60} , this should nevertheless provide a reasonable estimate for E_F . (In this derivation we use $k_F = 3.4$ nm, based on one electron per alkali dopant.) Such a low value of E_F may not rule out a phonon mechanism, but it does suggest that one should seriously consider how the coulomb repulsion of two electrons could be overcome to form pairs of electrons in such a narrow band. (It may be that the large size of the C_{60} molecule allows the on-site coulomb repulsion to be relatively small.) For the C_{60} molecule the frequencies of the vibrational modes range from $\omega_0 \approx 30\text{ meV}$ (250 cm^{-1}) to $\sim 250\text{ meV}$ ($2,000\text{ cm}^{-1}$), and thus many of the higher-frequency modes actually seem to lie above E_F , in contrast to the conventional situation (for example, in Pb and Al), where $E_F/\omega_0 \gg 1$.

An intimately related issue for the alkali fullerenes is the width of the conduction band. Using an assumed value for the energy gap and a measured coherence length, Holzner *et al.*²² have suggested a bandwidth of ~ 20 meV for K_3C_{60} . Erwin *et al.*²³ on the other hand, have calculated a bandwidth of ~ 600 meV, and thereby inferred a superconducting energy gap of $2\Delta \approx 17kT_c$ based on the relationship between ξ_0 , 2Δ and v_F . Even broader bandwidths (~ 1 eV) have been derived from angle-integrated photoemission measurements²⁴, although more recent angle-resolved measurements suggest a narrow bandwidth (≤ 100 meV) for certain occupied levels in the pure C_{60} solid²⁵. Recently Tycko *et al.*²⁶ have inferred from NMR (nuclear magnetic resonance) data a density of states at E_F of $\sim 20\text{ eV}^{-1}$ per

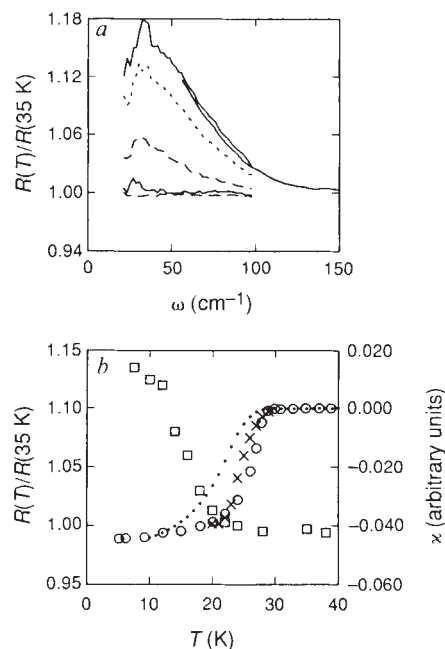


FIG. 2 a, Reflectivity of Rb_3C_{60} at 8, 12, 18, 24 and 38 K (solid, dotted, dashed, solid, dashed) divided by that at 35 K. b, Amplitude of R_s/R_n at $\omega = 50\text{ cm}^{-1}$ as a function of temperature ($\square$), along with the susceptibility (left-hand axis) of our epoxy-sealed sample before ($\times$) and after ($\bullet$) the infrared measurements, and of a powder specimen from the same batch ($\circ$).

C_{60} for K_3C_{60} , which corresponds to a bandwidth of ~ 200 meV. As a crude approximation one can estimate the bandwidth to be twice the Fermi energy, as the conduction bands are half-filled for Rb_3C_{60} . Our value of $E_F \approx 100$ meV then corresponds to a bandwidth of ≈ 200 meV. This is reasonably close to the value inferred from the NMR results and from angle-resolved photoemission data²⁵, but smaller than the results of refs 23 and 24. A possible resolution of the discrepancy between the narrow bandwidth inferred from low-energy measurements (NMR, infrared and critical field), and the broader bandwidth obtained by angle-integrated photoemission spectroscopy is discussed by Shen *et al.*²⁵

Thus far we have been considering the frequency scale of the infrared reflectivity changes. The amplitude of the enhancement, $R_s/R_n - 1$, is also useful, as it is related to the d.c. resistivity, ρ_{dc} , of Rb_3C_{60} . This is of particular interest in the present case because of the difficulties in using standard d.c. methods (A.F. Hebard, personal communication). At sufficiently low frequency ($\omega \approx 2\Delta$ and $\omega < 1/\tau$, where $1/\tau$ is the elastic scattering rate due to impurities) this relationship can be expressed as $\rho_{dc} = (\pi R_n^2 / 2\omega n^2) (R_s/R_n - 1)^2$ where n is the index of refraction of the sapphire, which is about 3.1 in the far-infrared. Because R_n is always close to 1 in the far infrared, the dependence of the right-hand side on R_n is dominated by the second term, $(R_s/R_n - 1)^2$, reflecting the fact that a material with higher resistivity has a lower normal-state reflectivity and thus a larger maximum value for $R_s/R_n - 1$. From this equation and the data in Fig. 1c for $\omega \approx 40$ cm⁻¹, we infer a d.c. resistivity of ~ 0.4 m Ω cm, which corresponds to a scattering rate of ~ 150 – 300 cm⁻¹. The large value of the scattering rate (relative to kT) indicates that it is associated with elastic scattering (disorder). Because scattering due to disorder tends to be strongly pair-breaking for non-zero angular momentum pairing, such a large scattering rate suggests that the pair wavefunction in Rb_3C_{60} is likely to have s -wave symmetry, in agreement with the more direct inference of Uemura *et al.*²⁷ based on the temperature dependence of the penetration depth.

Because this scattering rate is larger than the relevant infrared measurement frequencies, infrared and d.c. measurements of ρ_{dc} should agree. We find, however, that the infrared value is about an order of magnitude smaller than the estimates obtained from direct d.c. measurements (A. F. Hebard, personal communication). Presumably this difference is due to the length scale at which one is probing, relative to the grain size of the material (~ 50 nm for our material). The infrared measurements probe at a length scale of roughly v_F/ω , which is about 10 nm for $\omega \approx 40$ cm⁻¹, whereas the relevant length scale for d.c. measurements is the distance between contacts, which is much longer. On this basis one would expect the infrared result to be closer to the intrinsic value than the contact-based measurement. With the infrared value ($\rho_{dc} \approx 0.4$ m Ω cm), a mean free path, l , of ~ 2 nm is obtained, whereas from the higher d.c.-based estimates of ρ_{dc} , mean free paths of $l \leq 0.4$ nm have been inferred. (To get this result one can use $l = 3\pi^2 (\hbar/e^2) \sigma_{dc}/k_F^2$, where σ_{dc} is the d.c. conductivity). This difference is significant for two reasons: (1) the d.c.-based values of l suggested a mean free path less than the separation between C_{60} molecules, in apparent violation of the Ioffe-Regel guideline, whereas the longer infrared-based value does not, and (2) the d.c.-based value was so short as to suggest a substantial difference between ξ and ξ_0 , which would significantly affect the derivation of v_F , whereas the longer infrared-based value is consistent with only a modest difference between ξ and ξ_0 , as used above.

Although the details cannot be discussed here, the above estimates of resistivity and energy gap can be used to estimate the electromagnetic penetration depth, λ of Rb_3C_{60} . To do this one can use $\lambda = c/(8A)^{1/2}$ with the dirty limit formula $A \approx 2.2(2\Delta)/\rho_{dc}$, which is appropriate because the disorder-related scattering rate is large relative to 2Δ . ('Dirty' means that the elastic scattering rate is significantly larger than the gap, 2Δ , or,

equivalently, that the elastic mean free path is less than the coherence length.) With the above values of 2Δ and ρ_{dc} this equation implies a penetration depth of 500 ± 100 nm, which is larger than the value expected in the clean limit, but in agreement with estimates based on muon spin resonance²⁷. □

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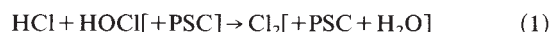
More rapid polar ozone depletion through the reaction of HOCl with HCl on polar stratospheric clouds

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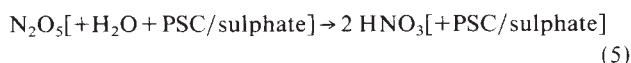
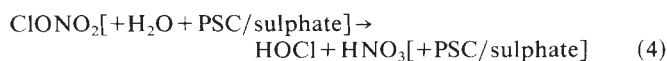
THE direct reaction of HOCl with HCl, known to occur in liquid water¹ and on glass surfaces², has now been measured on surfaces similar to polar stratospheric clouds^{3,4} and is shown here to play a critical part in polar ozone loss. Two keys to understanding the chemistry of the Antarctic ozone hole^{5–7} are, one, the recognition that reactions on polar stratospheric clouds transform HCl into more reactive species denoted by ClO_x (refs 8–12) and, two, the discovery of the ClO-dimer (Cl_2O_2) mechanism that rapidly catalyses destruction of O_3 (refs 13–15). Observations of high levels of OCIO and ClO in the springtime Antarctic stratosphere^{16–19} confirm that most of the available chlorine is in the form of ClO_x (refs 20, 21). But current photochemical models^{22,23} have difficulty converting HCl to ClO_x rapidly enough in early spring to account fully for the observations^{5–7,20,21}. Here I show, using a chemical model, that the direct reaction of HOCl with HCl provides the missing mechanism. As alternative sources of nitrogen-containing oxidants, such as N_2O_5 and $ClONO_2$, have been converted in the late autumn to inactive HNO_3 by known reactions on the sulphate-layer aerosols^{24–27}, the reaction of HOCl with HCl on polar stratospheric clouds becomes the most important pathway for releasing that stratospheric chlorine which goes into polar night as HCl.

The reaction



proceeds rapidly^{3,4} on both cold water-ice and solid nitric acid trihydrate, but is expected to be slow on sulphuric acid aerosols

unless they are dilute or partially neutralized^{3,30}. It probably proceeds through the adsorption of HCl or both species onto the surface, as do the other established heterogeneous reactions that return the adsorbed HCl to the gas phase in a more reactive form^{9,10,29-33}. This currently used set of heterogeneous reactions has been measured on both ice (polar stratospheric cloud, PSC, type II) and nitric acid trihydrate (PSC type I).



Experimental data for reactions (2) and (4) suggest^{3,4} that the HOCl released from reaction (4) on ice reacts rapidly on the surface with HCl, and thus reaction (2) may be thought of as a two-step process: reaction (4) followed by (1). In either case, the existence of pathway (1) makes the direct effects of PSC chemistry more straightforward: the competition for ClONO₂ between reactions (2) and (4) is no longer important, because either path (2) or (4) + (1) results in the conversion of HCl to Cl₂.

The partitioning of chlorine species as the Antarctic goes into polar night is unknown and represents a large uncertainty. Photochemical models indicate that HCl should be the dominant form of chlorine in the wintertime lower stratosphere, but we lack corroborating measurements. Here I first examine how the chlorine partitioning might be set in late autumn as photolytic activity ceases, and then focus on the photochemical evolution of ClO_x (ClO + 2 × Cl₂O₂ + Cl + 2 × Cl₂ + OClO) as the Antarctic vortex comes out of winter in the presence of PSCs (that is, the initial development of the ozone hole from August to October).

Reactions (4) and (5) occur in the laboratory on the surfaces of sulphuric acid/water mixtures²⁴⁻²⁷, which are ubiquitous in the lower stratosphere throughout the year. (PSCs are present only during the coldest parts of winter and early spring.) These

reactions have already been incorporated in models of ozone depletion for different concentrations of chlorine and background sulphate aerosols³⁴⁻³⁷. The photochemical evolution of an air parcel at 75° S from equinox (18 March) to late autumn twilight (20 May) is shown in Fig. 1 for cases with and without reactions (4) and (5). With gas-phase chemistry alone, most of the chlorine evolves into HCl with the remainder, ~30%, in ClONO₂; negligible amounts of HOCl and ClO_x survive. In this case high concentrations of N₂O₅ and NO_x (which represents NO + NO₂ + NO₃) are predicted, in contradiction to most polar observations³⁸. If sulphate-layer reactions (4) and (5) are included, they push most of the odd-nitrogen into HNO₃ as expected, and are also important in chlorine chemistry. Compared with gas-phase chemistry, both ClONO₂ and HCl are reduced at the end of autumn as HOCl and ClO_x species increase.

By the end of polar night, PSCs (type I or II) are assumed to have completely processed the chlorine and odd-nitrogen species according to reactions (2) and (3) above, leaving 1.2 p.p.b. residual HCl. In addition, the air has become denitrified and dehydrated as observed in the Airborne Antarctic Ozone Experiment³⁹. Whereas ClONO₂ (0.3 p.p.b.) can oxidize an equivalent amount of HCl, the amount of chlorine in the form ClO_x (0.1 p.p.b.) cannot. The HOCl (0.6 p.p.b.) created during polar night would reduce the residual HCl to 0.6 p.p.b. if we include reaction (1). In either case, gas-phase or sulphate-layer chemistry in the spring, the concentration of HCl carried into winter is higher than that of chlorinated oxidants. Only in the gas-phase case (ruled out here) can the residual N₂O₅ (1.2 p.p.b.) completely convert HCl to ClO_x through reaction (3).

The same photochemical model, now including PSC reactions (1) to (3), continues to follow the evolution of the isolated air parcel at 75° S latitude and 20 km altitude as it comes out of polar night from 29 July (noontime solar zenith angle of 94°) to 14 October (noontime solar zenith angle of 67°). The initial proportions and trends of the principal chlorine species are given in Fig. 1. The air parcel is assumed to be in contact with PSCs throughout the period, and two different chemistries are

FIG. 1 Noon-time mixing ratios of selected species from the photochemical evolution of an isolated air parcel at 75° S, 20 km altitude. The model was initialized on 18 March (volume mixing ratios O₃ = 2.5 × 10⁻⁶, HNO₃ = 5 × 10⁻⁹, NO_x = 2 × 10⁻⁹, ClONO₂ = 1 × 10⁻⁹, HCl = 1.5 × 10⁻⁹, BrO = 15 × 10⁻¹², H₂O = 4 × 10⁻⁶, H₂ = 0.5 × 10⁻⁶, CH₄ = 1 × 10⁻⁶) and integrated through the autumn, with solar declination changing roughly every 5 days. Two cases, 'gas-phase' and 'sulphate', are shown. For the heterogeneous reactions (4) and (5) on sulphate-layer aerosols, I adopt reaction probabilities of 0.01 and 0.1, respectively, with surface area ~2.5 × 10⁻⁸ cm² cm⁻³. The model was re-initialized on 29 July with conditions (O₃ = 2.5 × 10⁻⁶, H₂O = 2 × 10⁻⁶, HNO₃ = 2 × 10⁻⁹, NO_x = ClONO₂ = 0, HCl = 1.2 × 10⁻⁹, ClO_x = 0.7 × 10⁻⁹, HOCl = 0.6 × 10⁻⁹ (denoted 'winter HOCl') and with alternative conditions (HOCl = 0, ClO_x = 1.3 × 10⁻⁹) with results shown in Table 1. The 'standard' PSC chemistry reactions (2) and (3) were integrated through the spring at their effective rate limits, $k = 1 \times 10^{-11} \text{ cm}^3 \text{ s}^{-1}$, yielding maximum conversion to ClO_x (see text and Table 1). The 'new' PSC chemistry includes in addition reaction (1). The photochemical model is documented in refs 43, 44. The calculations use climatological mean temperatures and ozone profiles for the photolysis rates. I assume an atmosphere of 1,000 mbar, a surface albedo of 0.30, fully spherical solar extinction, and Rayleigh-phase scattering in a plane-parallel atmosphere.

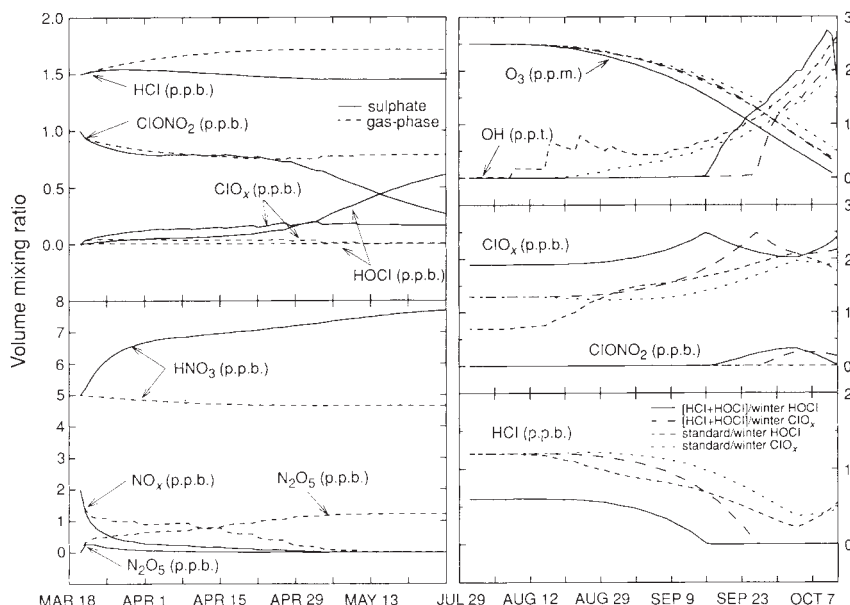


TABLE 1 Chemical rate limits

Reaction (1)*	
$O(^1D) + H_2O (\times 2)$	$44 \text{ cm}^{-3} \text{ s}^{-1}$
$O(^1D) + H_2 (\times 2)$	5
CH_4 oxidation cycle	505
HNO_3 + photon	171
$HNO_3 + OH (\times -1)$	-8
net production of HO_x	$725 \text{ cm}^{-3} \text{ s}^{-1}$
$HO_2 + ClO \rightarrow HOCl + O_2$	720
$HOCl + HCl(PSC)$	720
24-h average OH	$5.8 \times 10^3 \text{ cm}^{-3}$
24-h average HO_2	$3.2 \times 10^4 \text{ cm}^{-3}$
Reaction (2)†	
HNO_3 + photon	$171 \text{ cm}^{-3} \text{ s}^{-1}$
$HNO_3 + OH$	248
net production of NO_x	$419 \text{ cm}^{-3} \text{ s}^{-1}$
$ClONO_2 + HCl(PSC)$	418
24-h average OH	$1.9 \times 10^5 \text{ cm}^{-3}$
24-h average HO_2	$4.0 \times 10^5 \text{ cm}^{-3}$

Latitude 75°S at 20 km with solar declination of $+6^\circ$ (10 September).

* In the saturation limit $k_{HCl+HOCl} > 1 \times 10^{-12} \text{ cm}^3 \text{ s}^{-1}$.

† In the saturation limit $k_{HCl+ClONO_2} > 5 \times 10^{-13} \text{ cm}^3 \text{ s}^{-1}$.

considered: a 'standard' model with reactions (2) and (3), and a 'new' model that also includes reaction (1). The initial conditions, taken from the March–May calculations, include a large abundance of HOCl ('winter HOCl'), which in the 'new' model rapidly oxidizes a large fraction of the HCl. For a better comparison of the 'standard' and 'new' PSC chemistries, I also include an initialization without HOCl, in which the HOCl generated from reaction (4) during polar night is arbitrarily converted to ClO.

Limits to the effectiveness of PSC chemistry are found through a sensitivity study that identifies the key gas-phase chemical rates responsible for liberation of ClO_x . I treat the PSC reactions (1) to (3) as effective two-body rates with coefficients k_1 , k_2 and k_3 , ranging from 0 to $1 \times 10^{-11} \text{ cm}^3 \text{ s}^{-1}$. In September, the effect of reaction (2) alone is negligible for $k_2 < 1 \times 10^{-15} \text{ cm}^3 \text{ s}^{-1}$ and saturates for $k_2 > 5 \times 10^{-13} \text{ cm}^3 \text{ s}^{-1}$. This range of two-body rates corresponds to reaction probabilities for $ClONO_2$ of ~ 0.001 – 0.5 for typical PSC surface areas⁴⁰ of $2 \times 10^{-7} \text{ cm}^2 \text{ cm}^{-3}$ and 1 p.p.b. of HCl. In the upper limit, oxidation of HCl is maximal and is limited by the production of $ClONO_2$, which is in turn limited by release of NO_x from destruction of HNO_3 by photons or OH reactions. Critical rates in this limit are summarized in Table 1.

Reaction (1) has considerable impact on the HO_x chemistry, reducing OH and HO_2 concentrations by 40% even for k_1 as low as $1 \times 10^{-16} \text{ cm}^3 \text{ s}^{-1}$. Its effect saturates for $k_1 > 1 \times 10^{-12} \text{ cm}^3 \text{ s}^{-1}$, and the rate of reaction (1) is limited by the production of $HO_x(OH + HO_2 + 2 \times H_2O_2)$ because HOCl is produced by reaction of HO_2 with ClO and reaction (1) is the dominant sink for HO_x . Most HO_x is produced by primary reactions of $O(^1D)$ with H_2O , H_2 and CH_4 , followed by amplification through the CH_4 oxidation cycle (for example, one OH begets two or more HO_2 as CH_4 is oxidized to CO); but an important fraction can come from HNO_3 photolysis. The rate-limiting reactions are shown in Table 1.

In the calculations shown in Fig. 1, the upper limits for PSC reaction rates are used, and thus the increase in ClO_x and the corresponding decrease in O_3 are the largest possible, being limited by the amount of sunlight. The densities of N_2O_5 and NO_x remain low throughout this period because of the effectiveness of reaction (2): any odd-nitrogen not tied up as HNO_3 is in the form of $ClONO_2$. Therefore reaction (3), even at PSC

rates, is unimportant. Similarly, reactions (4) and (5), continued as sulphate-layer chemistry, are not important.

With the 'new' PSC chemistry (reaction 1), concentrations of ClO_x (excepting the initial reaction of winter HOCl) begin to increase by 26 August and continue for 3–4 weeks until the HCl supply is exhausted. Both OH and $ClONO_2$ concentrations are suppressed until the HCl disappears (see below). Ozone losses become constant, ~ 50 – 60 p.p.b. per day, independent of the O_3 abundance. This unusual property of Cl_2O_2 -catalysed ozone loss occurs when ClO_x is the dominant chlorine species and thus Cl_2O_2 densities are independent of O_3 densities; it allows for the more rapid linear loss, rather than exponential decay, to less than 0.2 p.p.m. O_3 as observed⁷. With the 'standard' PSC chemistry (reaction 2), increases in ClO_x (again excepting the early photolysis of winter HOCl) do not begin until 9 September and never reach values as high as with reaction (1). The rapid loss of O_3 is delayed by five days, and ClO_x concentrations begin to decline as HCl recovers in October.

Concentrations of OH and HO_2 are an important diagnostic of the proposed mechanism, because OH is suppressed by reaction (1). In contrast, reaction (2) has little effect on HO_x : noontime OH concentrations climb to $1 \times 10^6 \text{ cm}^{-3}$ in the first week of September, and without reaction (1) the winter HOCl provides a pulse of OH in early August. Concentrations of HOCl or $ClONO_2$ are poor tests of the proposed mechanisms: in the saturation limit, the rate of HCl oxidation is independent of the PSC activity, but the densities of HOCl and $ClONO_2$ are inversely proportional to PSC surface area. The low column-abundances of HOCl observed in late September⁴¹ are consistent with all of these calculations, in which noontime HOCl densities range from 1 to $3 \times 10^8 \text{ cm}^{-3}$.

The timing of the rapid ClO_x increase in these calculations is appropriate to the mean insolation at 75°S . At a latitude of 70°S , the increase in sunlight would occur about 12 days earlier. During this chemical evolution, one expects that the air parcels remain neither isolated nor at one latitude. Lagrangian trajectories show latitudinal excursions⁴², and wind shear plus molecular diffusion will lead to mixing of parcels⁴³. Neither of these processes would affect the basic results shown here, but they might alter the mean insolation, or effective latitude of the air parcel.

The oxidation of HCl by HOCl (reaction 1) always exceeds that by $ClONO_2$ (reaction 2), often by more than a factor of 2. Furthermore, the HOCl pathway becomes effective at 75°S by the end of August, well before the $ClONO_2$ mechanism. The upper limit of ClO_x growth by reaction (2) is almost linearly proportional to the gas-phase abundance of HNO_3 ; but if HNO_3 is tied up as solid $HNO_3 \cdot 3H_2O$ (type I PSCs) and cannot be photodissociated, then reaction (2) becomes ineffective. A simple cycling of HNO_3 between PSCs and gas phase due to parcel trajectories⁴⁴ can circumvent this impasse, but would further lengthen the time needed to oxidize HCl.

Heterogeneous reactions involving HOCl are therefore important in creating high concentrations of ClO_x , and hence O_3 losses, during early spring in the polar stratosphere. The oxidation of HCl into forms of ClO_x requires that PSCs be prevalent, but the growth in ClO is limited by the photochemical production of the oxidant (HOCl or $ClONO_2$) and cannot be accelerated beyond this limit by known microphysical processes on stratospheric clouds. □

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Link between iron and sulphur cycles suggested by detection of Fe(II) in remote marine aerosols

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IRON is essential to the growth of organisms, and iron derived from the atmosphere may be the limiting nutrient for primary productivity in some oceanic regions^{1–6}. Aeolian mineral dust is the chief source of marine iron in many areas^{1–3,5,7}, but there is little information on the chemical form of the iron in this dust. Here we report that Fe(II) contributed $56 \pm 32\%$ of the total iron in marine aerosol samples collected over the central North Pacific and $49 \pm 15\%$ at Barbados. We suggest that the key reaction that produces Fe(II), and hence increases the solubility of marine aerosol iron in sea water, is $[\text{Fe(II)}(\text{OH})(\text{H}_2\text{O})_5]^{2+} + \text{H}_2\text{O} \xrightarrow{h\nu} [\text{Fe(II)}(\text{H}_2\text{O})_6]^{2+} + \text{OH}^\cdot$ (refs 8–10). The presence of Fe(II) in remote marine aerosols suggests that the OH radical has been produced in these heterogeneous reactions. From consideration of both the marine biological production of dimethylsulphide and the subsequent oxidation of reduced forms of sulphur in the atmosphere, we propose that the iron and sulphur cycles in both the atmosphere and the ocean may be closely coupled.

We collected atmospheric aerosol samples continuously for one-week periods during 1986 using high-volume air-sampling systems and Whatman 41 filters (20×25 cm) at four North

Pacific island stations (Midway, Oahu, Enewetak and Fanning). We also collected aerosol samples at Barbados in the North Atlantic in September and October 1988 and April 1990. Urban aerosol samples were collected during November 1989 in the city of Xian (34° N, 109° E) in central China near the loess plateau region. Samples of loess were collected at Luochuan (35.5° N, 109° E), China, in the loess plateau region, an important source area for the mineral aerosol particles found over the North Pacific. All samples were stored in the dark and frozen from the time of collection until analysis.

Iron(II) concentrations in aerosols and Chinese loess were determined by high-performance liquid chromatography (HPLC)¹¹. A considerable proportion of the iron in the aerosols was in the form of Fe(II) (see Fig. 1). Concentrations of Fe(II) over the North Pacific ranged from 11% to nearly 100% of the total iron, $T(\text{Fe})$. The volume-weighted mean Fe(II) concentration was $56 \pm 32\%$ of $T(\text{Fe})$ at the North Pacific sites and $49 \pm 15\%$ at Barbados. The $T(\text{Fe})$ at Barbados was $\sim 0.6\text{--}5 \mu\text{g m}^{-3}$, whereas $T(\text{Fe})$ at the North Pacific sites was $\sim 0.010\text{--}0.15 \mu\text{g m}^{-3}$. Although the percentage of Fe(II) in some of the Barbados samples was less than 10%, the Fe(II) concentrations were in the range of $\sim 28\text{--}150 \text{ ng m}^{-3}$, similar to the Fe(II) concentration range of $5\text{--}135 \text{ ng m}^{-3}$ in the North Pacific samples.

The urban aerosol samples from Xian contained 4–11% Fe(II), with a volume-weighted average of $5 \pm 3\%$. (In urban fog samples from Zurich, 20–90% of the total iron was present as Fe(II)¹².) Samples of Chinese loess contained only $0.4 \pm 0.3\%$ Fe(II). During atmospheric transport over $\sim 10,000 \text{ km}$ from central Asia to the central North Pacific, the Fe(II) fraction of $T(\text{Fe})$ increased from less than 1% in the Chinese loess to more than 50% in the marine aerosols (see Table 1). The solubility of iron in these same marine aerosols in acidified water (pH = 2.0–5.6) was 5–17 times higher than the iron in Chinese loess¹¹.

Is the Fe(II) in remote marine aerosols simply small-particle Fe(II) produced in the source area, which becomes increasingly important as the large particles are lost during long-range transport, or is it produced by transformation processes during the long-range transport? Lead can be used as a reference to compare with Fe(II), as lead over the North Pacific is derived largely from anthropogenic sources in Asia and is found primarily on small, submicrometre aerosol particles¹³. Lead in a typical urban area such as Beijing¹⁴ ranged from 52 to 257 ng m^{-3} . Observed lead concentrations over the central North Pacific ranged from a mean of 2 ng m^{-3} at Oahu¹⁵ to $\sim 0.33\text{--}1.1 \text{ ng m}^{-3}$ between 20° and 50° N (ref. 16). This indicates that lead, representative of small particles, decreased by a factor of more than 100 during

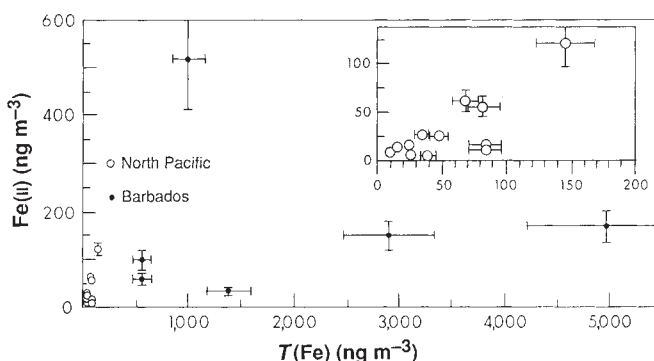
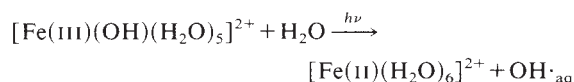


FIG. 1 Fe(II) concentration as a function of total iron concentration, $T(\text{Fe})$, in remote marine aerosols from islands in the North Pacific and from Barbados in the North Atlantic. The peak of the Fe(II) complex with ferrozine (FZ), $[\text{Fe(II)}(\text{FZ})_3]^{2-}$, in the HPLC measurement was identified by retention time, by the addition of known quantities of standard and by comparing the peak ratios at various wavelengths.

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its long-range transport from urban areas in China to the North Pacific. The Fe(II) concentration in Xian urban aerosols ranged from 120 to 210 ng m⁻³, and the Fe(II) concentrations in remote marine aerosols were 15–56 ng m⁻³ at Oahu and 16–135 ng m⁻³ at Midway. These are rather similar ranges, suggesting that even if all of the Fe(II) in the source area was present in small particles, only a small proportion of the Fe(II) in the remote marine aerosols could have been transported as Fe(II) from the source areas. This supports the hypothesis that there is considerable transformation of Fe(III) to Fe(II) during transport.

Photoreduction of Fe(III) has recently been proposed¹⁰ as a principal source of OH· radicals in clouds, fog and rain:



Faust and Hoigne¹⁰ determined that the quantum efficiency for this photolysis reaction is 0.14 ± 0.04 at 313 nm, seven times higher than an earlier conservative estimate⁸ of 0.02. This may be the key reaction that produces Fe(II) in remote marine aerosols. The main Fe(III)-hydroxy complexes in water are pH-dependent, and their percentages relative to the total dissolved Fe(III) ion concentration at pH 0–8 are shown in Fig. 2a. At pH ~2.5–5, [Fe(III)(OH)(H₂O)₅]²⁺ is the dominant form, whereas at pH ≤ 2.5, [Fe(III)(H₂O)₆]³⁺ is the dominant species. The pH of rainwater is, in general, in the range 3.5–5.5, and the

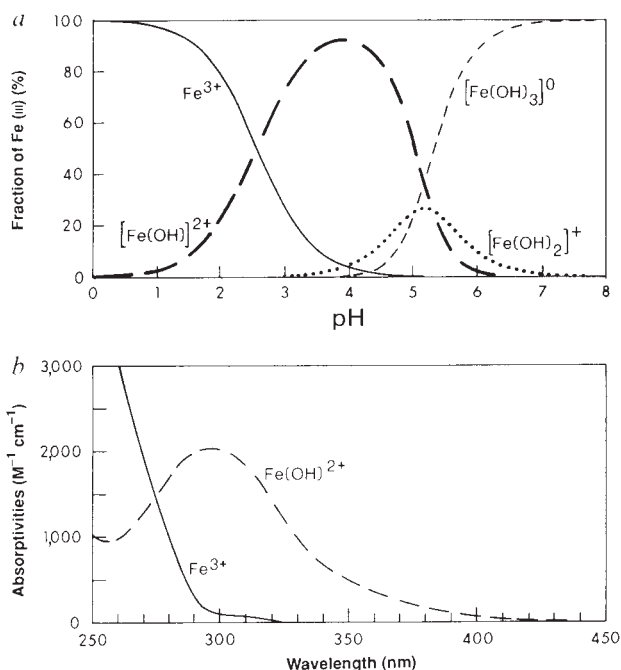
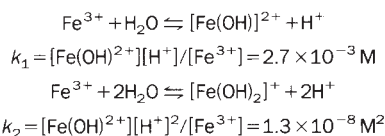
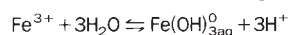


FIG. 2 a, Calculation of monomeric Fe(III)-hydroxy complexes as a function of pH is based on the following equilibria:



(k_1 and k_2 from ref. 10, at 293 K with ionic strength 0.03 M)



$$k_3 = [\text{Fe(OH)}_3^0][\text{H}^+]^3/[\text{Fe}^{3+}] = 1.2 \times 10^{-13} \text{ M}^3 \text{ at ionic strength 0.03 M}$$

(k_3 was taken from a value of 2.4×10^{-14} (ref. 25) in sea water at 36.22‰ salinity and 298 K, with a correction for ionic strength effects from 0.7 M to 0.03 M). b, Fe(III)-based decadic molar absorptivities of Fe³⁺ and Fe(OH)²⁺. Adapted from ref. 10.

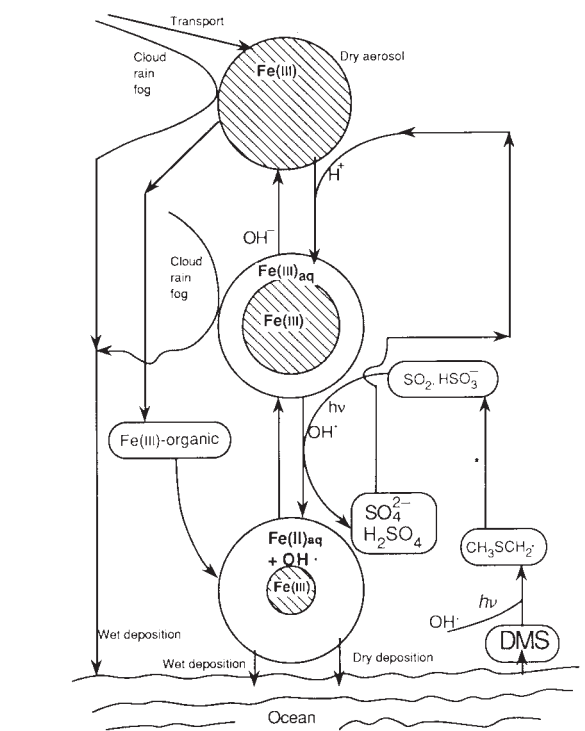


FIG. 3 Transformation scheme for iron in remote marine aerosols. The step marked with a star (*) indicates a multistep process:



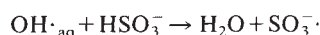
average pH of cloud droplets in non-urban areas is typically ~3.5 (ref. 17) or perhaps a little lower. But the pH of a marine aerosol solution at ambient relative humidities could be lower than 1.0 (ref. 18). The photoreduction of [Fe(III)(H₂O)₆]³⁺ in the atmosphere is not a principal source of OH· because its absorption spectrum does not significantly overlap the solar spectrum^{8,10} (see Fig. 2b). Therefore, if the aerosols did not pass through clouds during their long-range transport, the primary source for Fe(II) would be the photoreduction of [Fe(III)(H₂O)₆]³⁺, which would be minimal. This would mean that the fraction of Fe(II) might be much less in these particles than in particles that are scavenged by cloud or rain droplets during their transport, possibly explaining why the Fe(II) percentage of T(Fe) in remote marine aerosols varies widely. The percentage of Fe(II) in aerosols might even indicate how often and for how long aerosols are included in cloud processes during their long-range transport. It is possible, of course, for some of the Fe(II) to be re-oxidized to Fe(III) in the aerosol particles during transport^{19,20}, and it is likely that a pseudo-steady-state concentration ratio between Fe(II) and Fe(III) may develop, with the ratio determined both by the pH range over which the particles have been exposed and by the availability of appropriate oxidizing species for Fe(II).

Charlson *et al.*²¹ suggested the possible existence of a climate feedback loop produced by the oxidation of dimethyl sulphide, DMS, emitted from the open ocean, with subsequent formation of sulphate cloud condensation nuclei. One very important step in this climate feedback loop of Charlson *et al.*²¹ is the heterogeneous oxidation of S(IV) to sulphate, and this step may be closely related to the Fe(III)-hydroxy complex reduction^{8,9,22} through the formation of OH·. Atmospheric iron present in remote marine aerosols may thus be involved in two important environmental processes. First, its transformation to the relatively soluble Fe(II) during its long-range transport makes iron more readily available to plankton in surface waters of the open

TABLE 1 Percentage of total iron represented by Fe(II)

Sample type	Fe(II) (%)
Chinese loess	0.4 ± 0.3
Xian aerosols	5 ± 3
Pacific aerosols	56 ± 32

ocean. If iron is the limiting nutrient in certain areas of the ocean, it may then affect the planktonic production of DMS in those waters. Second, reduction of atmospheric Fe(III) may be a significant source of the key oxidant OH·, which may have an important role in the oxidation of reduced sulphur in the atmosphere, for example, through an initiation step involving the oxidation of bisulphite to the sulphite free radical^{23,24}.



Therefore, there are two potential positive feedbacks relative to the production of Fe(II) and OH·. Atmospheric dust provides Fe(III), which when reduced in the aerosol particles can provide OH· and Fe(II). Then, more Fe(II) could result in the marine production of more DMS, and the increased DMS provides more SO₂ and acidic SO₄²⁻ aerosol, which in turn leads to more dissolved Fe(III); and more OH· could oxidize more reduced sulphur and thus result in more acidic SO₄²⁻ aerosol and, again, more dissolved Fe(III). Both of these could again lead to additional Fe(II) and OH·, and so on. These feedbacks might affect

climate and/or primary biological productivity in some areas of the open ocean. The proposed transformation mechanisms for atmospheric iron and their relation to OH· and atmospheric sulphur during long-range transport are illustrated in Fig. 3. □

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Evolution of king crabs from hermit crab ancestors

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KING crabs (Family Lithodidae) are among the world's largest arthropods, having a crab-like morphology and a strongly calcified exoskeleton¹⁻⁶. The hermit crabs, by contrast, have depended on gastropod shells for protection for over 150 million years^{5,7}. Shell-living has constrained the morphological evolution of hermit crabs by requiring a decalcified asymmetrical abdomen capable of coiling into gastropod shells and by preventing crabs from growing past the size of the largest available shells¹⁻⁶. Whereas reduction in shell-living and acquisition of a crab-like morphology (carcinization) has taken place independently in several hermit crab lineages, and most dramatically in king crabs¹⁻⁶, the rate at which this process has occurred was entirely unknown^{2,7}. We present molecular evidence that king crabs are not only descended from hermit crabs, but are nested within the hermit crab genus *Pagurus*. We estimate that loss of the shell-living habit and the complete carcinization of king crabs has taken between 13 and 25 million years.

Although the asymmetrical abdomens of the king crabs have long been considered evidence of hermit crab ancestry^{1,2,4,6}, phylogenetic studies of larval and adult morphology have disagreed about the relationships of king and hermit crabs^{4,8-10}. Furthermore, the absence of fossil king crabs has made it impossible to determine how long ago this group has diverged^{2,7}. By contrast, the hermit crab genus *Pagurus* is considered to be

ancient, with fossils being assigned to the genus as early as the Lower Cretaceous (>113 Myr⁷). A strongly supported phylogeny based on DNA sequences from the gene encoding mitochondrial large-subunit ribosomal RNA amplified by the polymerase chain reaction (Table 1) places two Alaskan genera of the king crabs not only within the Family Paguridae, but within the genus *Pagurus* (Fig. 1).

The minimum length phylogenetic tree remains the same whether or not the analysis was carried out using parsimony or distance techniques, and the next most parsimonious tree is four steps longer (Fig. 1). The 108 phylogenetically informative positions represent a sufficiently large sample size to achieve high levels of significance using commonly used statistical tests: all but one node is supported by >96% in bootstrap analysis (Fig. 1), and the most parsimonious tree supporting the monophyly of the Family Paguridae with respect to the Family Lithodidae is eight steps longer than the minimum length tree, a difference that is highly significant ($P < 0.005$; Fig. 1).

Apart from the placement of the king crabs within the genus *Pagurus*, the molecular phylogeny presented here is in accord with the conclusions of morphological taxonomy, and particularly with studies that have considered larval morphology^{4,8,9}. Previous workers have questioned the monophyly of the genus *Pagurus*, but only with respect to other hermit crabs^{9,11}. Furthermore, the molecular data agrees with morphological conclusions in finding strong support for the sister group relationships of the morphologically similar species *P. acadianus* and *P. bernhardus*¹², of the 'left-handed' hermit crab genera *Clibanarius* and *Coenobita*^{4,10}, and of the geographically disjunct populations of *Pagurus longicarpus* and *P. pollicaris*^{12,13} (Fig. 1).

A highly significant correlation between geological time and divergence of DNA sequences from the gene encoding mitochondrial large-subunit rRNA within the hermit crab lineage allows the date of the transformation of king crabs from hermit crabs to be estimated using conservative methods of temporal scaling (19.4 ± 5.9 Myr, or mid-lower Miocene;

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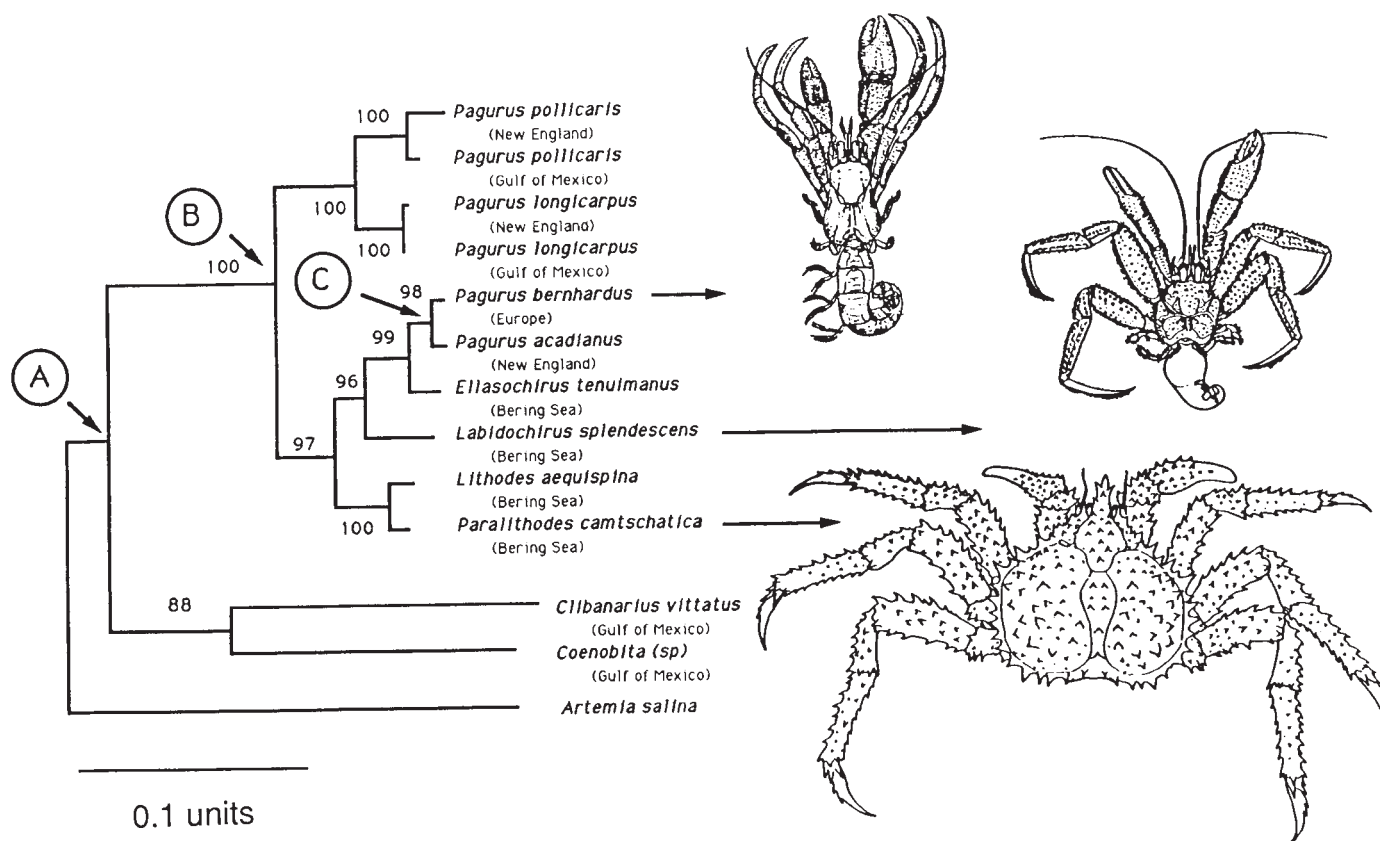
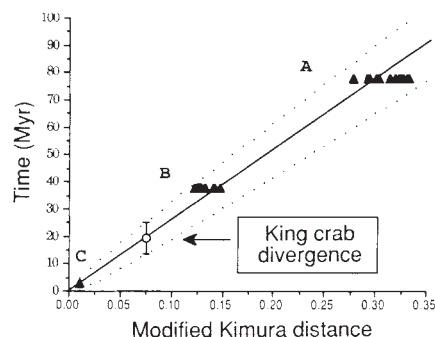


FIG. 1 Minimum-length phylogenetic tree obtained by parsimony and distance methods based on DNA sequences from the gene encoding mitochondrial large-subunit ribosomal RNA, with illustrations of various stages of carcinization from within a single monophyletic group: *Pagurus bernhardus* (after Borradaile¹) is a typical shell-living hermit crab that can fully retract into its gastropod shell; *Labidochirus splendescens* (after Hart²⁰) is a partially carcinized hermit crab displaying a reduced dependence on gastropod shells with only its abdomen protected by the shell¹; *Paralithodes camtschatica* (after Hart²⁰) is a fully carcinized king crab that never occupies a shell. Numbers refers to the percentage of times a node was supported in 500 bootstrap pseudoreplicates using PAUP 3.0q (ref. 24), and the letters A–C refer to nodes corresponding to the estimated dates of geological divergence described in Fig. 2. Branch lengths are shown as modified Kimura distances²⁵. Parsimony analysis with PAUP 3.0q found a single minimum-length tree 317

steps long with 108 phylogenetically informative characters. The next most parsimonious tree is four steps longer. The tree shown had a consistency index of 0.811 with and 0.721 without including uninformative characters. Modified Kimura distances were calculated taking into account base-pair composition using the DNADIST program of PHYLIP 3.3 (ref. 25), and identical minimum-length trees were obtained using PHYLIP 3.3 least-squares programs KITSCH (assuming) and FITCH (not assuming) a molecular clock. The tree shown is drawn with branch lengths calculated by the FITCH program. Sums of squares for this tree were 0.182 (FITCH) and 0.638 (KITSCH). The most parsimonious tree supporting the monophyly of the Family Paguridae is 8 steps longer than the minimum length tree; this difference is significant according to the version of Kishino and Hasegawa's²⁶ test performed by the DNAPARS program of PHYLIP 3.3 (ref. 25) (2.83 standard deviations or $P < 0.005$).

FIG. 2 Temporal scaling of genetic distance against three hypothesized geological dates of divergence, indicated by letters A–C, along with the estimated date of divergence for the king crab lineage. Dotted lines represent 95% confidence intervals for predictions of time based on a weighted linear regression forced through the origin. The equation of the line is $\bar{Y} = b\bar{X}$. The standard deviation of the residuals under this model of regression is $S_{Y,X} = \{[\sum (Y^2/X) - (\sum Y)^2/\sum X]/(n-1)\}^{0.5}$. The standard deviation of the predicted value of time ($\bar{Y}$) is given by $S_{\bar{Y}} = S_{Y,X}(X^2/\sum X^2 + X)^{0.5}$. The 95% confidence intervals for the predicted values of time ($\bar{Y}$) shown are calculated by multiplying $S_{\bar{Y}}$ by the critical value of the Student's t -distribution for 29 degrees of freedom (2.045). This procedure, and especially the consideration of time as the dependent variable, has been recommended by advocates of increased rigour in 'molecular clock' calibrations²⁷. Geologically determined dates of vicariance: A, divergence of 'left' and 'right'-handed hermit crabs (see Fig. 1). Right-handed hermit crabs (Family Paguridae) are known since the Jurassic, but the earliest known fossils of the left-handed family Diogenidae (which includes *C. vittatus*) are from the Upper Cretaceous of Texas (mid-Campanian, about 73–78 Myr^{5,7}). B, vicariance of Atlantic and Pacific cold-water marine faunas in the upper Oligocene and lower Eocene, about 35–40 Myr^{5,28,29} (see Fig. 1). C, vicariance of the amphiatlantic boreal marine province at the onset of northern hemispheric glaciation in the Pliocene, about 2.5–3.1 Myr¹² (see Fig. 1). Ages represent averages of the



geological ranges. Genetic distances were calculated as described in Fig. 1. The points shown are significantly correlated ($P < 0.001$; Spearman's coefficient of rank correlation). The predicted date of king crab divergence is calculated from the average of six pairwise distances between king crabs and their nearest hermit crab relatives according to Fig. 1.

TABLE 1 Alignment of DNA sequences from the gene encoding mitochondrial large-subunit rRNA

<i>Artemia salina</i>	TAATTAAAGG	GCCGTGGTAT	AXCTGACCAT	CGGAAGGTAG	CATAATCATT	AGCCTTTTGA
<i>Clibanarius vittatus</i>	GTG...G	...C	TTT...G	...	...	...T...A
<i>Coenobita</i> sp.	AT...G	...C	TT...G	...	...	...T...A
<i>Lithodes aequispina</i>	A...A	CA	TTX...TG	...	A A	T T...A
<i>Paralithodes camtschatica</i>	A...A	CA	TTX...TG	...	A A	T T...A
<i>Ellasochirus tenuimanus</i>	AT...A	CA	TCX...TG	...	A A	T T...A
<i>Labidochirus splendescens</i>	AT...A	CA	TTX...TG	...	A A	T T...A
<i>Pagurus bernhardus</i>	GT...A	CA	TCX...TG	...	A A	T T...A
<i>Pagurus acadianus</i>	GT...A	CA	TCX...TG	...	A A	T T...A
<i>Pagurus pollicaris</i> (NE)	G...A	CA	TTX...TG	...	AGA	T T...A
<i>Pagurus pollicaris</i> (GU)	G...A	CA	TTX...TG	...	AGA	T T...A
<i>Pagurus longicarpus</i> (NE)	...A	CA	TTX...TG	...	AGA	T G...A
<i>Pagurus longicarpus</i> (GU)	...A	CA	TTX...TG	...	AGA	T G...A
<i>Artemia salina</i>	TTTGAGGCTG	GAATGAATGG	TTTGAAGAGA	GATGGTCTGT	CTCXXTTTGA	TXTAATATGA
<i>Clibanarius vittatus</i>	...CA	...C	...G	A CACA	T AGXXAC	AAG T...
<i>Coenobita</i> sp.	...G	C T	...A	OGA...T	...A A	AGCAAA AAA
<i>Lithodes aequispina</i>	A AA	T	...A	G A AG T CA	T	AA A TT
<i>Paralithodes camtschatica</i>	A AA	T	...A	G A AG T CA	T	AA A TT
<i>Ellasochirus tenuimanus</i>	A GA	T	...A	G A AG T CA	T	AA A TT
<i>Labidochirus splendescens</i>	A GA	T	...A	G A AG T CA	T	AA A TT
<i>Pagurus bernhardus</i>	A G	T	...A	G A AG T CA	T	AA A TT
<i>Pagurus acadianus</i>	A G	T	...A	G A AG T CA	T	AA A TT
<i>Pagurus pollicaris</i> (NE)	A G G	A	...A	G A AG T C	T	AA A TT
<i>Pagurus pollicaris</i> (GU)	A G G	A	...A	G A AG T CA	T	AA A TT
<i>Pagurus longicarpus</i> (NE)	A G	...	...A	G A AG T CA	T	AA A TT
<i>Pagurus longicarpus</i> (GU)	A G	...	...A	G A AG T CA	T	AA A TT
<i>Artemia salina</i>	AGTTAATCTT	TAAGTAAAA	AGCTTAAATG	TACTTGGAGG	GCGATAAGAC	CCTATAGATC
<i>Clibanarius vittatus</i>	T...CT	...	G...	A T AAA A	A	...
<i>Coenobita</i> sp.	C...CT	A	G...T A	TTCA A A	A	...
<i>Lithodes aequispina</i>	T...G CT	...	G...A	A TCAAA A	A	...
<i>Paralithodes camtschatica</i>	T...G CT	...	G...A	A TCAAA A	A	...
<i>Ellasochirus tenuimanus</i>	T...G CT	C	G...G A	A T AAA A	A	...
<i>Labidochirus splendescens</i>	T...G CT	...	G...A	A T AAA A	A	...
<i>Pagurus bernhardus</i>	T...G CT	C	G...G A	A T AAA A	A	...
<i>Pagurus acadianus</i>	T...G CT	C	G...G A	A T AAA A	A	...
<i>Pagurus pollicaris</i> (NE)	T...G CT	...	G...A	A A AAA A	A	...
<i>Pagurus pollicaris</i> (GU)	T...G CT	...	G...A	A A AAA A	A	...
<i>Pagurus longicarpus</i> (NE)	T...G CT	...	G...A	A A AAA A	A	...
<i>Pagurus longicarpus</i> (GU)	T...G CT	...	G...A	A A AAA A	A	...
<i>Artemia salina</i>	TTTACATTTA	ATXTCTTTTG	TCTTGCGGTA	GXGTAATTAG	ACAGAGTAXX	XAAACAXXXX
<i>Clibanarius vittatus</i>	...XXXX	T A G	A TTTAA	TTGTTG GT	TTA AA GA	A T T AAGT
<i>Coenobita</i> sp.	...C XXXX	CCAC	AA CC	ATC GT GT	TXA G GA	AGT T AAAA
<i>Lithodes aequispina</i>	...A A	A A A	A TTA T	XTA G GA	TGATA TA	A T TAGG
<i>Paralithodes camtschatica</i>	...A A	A A A	A TTA CT	XTA G GT	T AGAA TA	A T TAGG
<i>Ellasochirus tenuimanus</i>	...A A	CA G C A	A TTA T	XTA GT	T A A TT	A TTXGTGA
<i>Labidochirus splendescens</i>	...A A	AGAC	A A TTA T	XTA G GT	GT AG TA	A TTXGTGA
<i>Pagurus bernhardus</i>	...A A	CA G C A	A TTA T	XTA G T	T A A TT	A TTXGTGA
<i>Pagurus acadianus</i>	...A A	CA G C A	A TTA T	XTA G GT	T A A TT	A TTXGTGA
<i>Pagurus pollicaris</i> (NE)	...AA	T A A A	AA TTAAGT	XTA TT	T A A TA	A GT TAGT
<i>Pagurus pollicaris</i> (GU)	...AA	T A A A	AA TTA T	XTA TT	T A A TA	A GT TAGT
<i>Pagurus longicarpus</i> (NE)	...AA	T A A A	AA TTAAGT	XTA TT	T A A TA	A GT TAGT
<i>Pagurus longicarpus</i> (GU)	...AA	T A A A	AA TTA T	XTA TT	T A A TA	A GT TAGT
<i>Artemia salina</i>	XXATGTTCCG	TTGGGGCGAC	GGTAAGAXXA	CAGAATAAAC	XACTTACAAAC	ATAACACAT
<i>Clibanarius vittatus</i>	AG...GT	...A	A T TA	T AG T G	TGT GGT A	T ACA A
<i>Coenobita</i> sp.	ACG...TT	...A	AAAG T TA	TTA X	TGT XXTT A	T TT A ACA
<i>Lithodes aequispina</i>	TTX...GC	C...G	TAG T TAX	XTA	TGTC T GT	T T T A
<i>Paralithodes camtschatica</i>	TTX...GC	C...G	TAG T TAX	XTA	TGTC T T	T T T A
<i>Ellasochirus tenuimanus</i>	ATX...TC	C...G	TAG T TAX	XTA	TGTC TTTA	T T T C G
<i>Labidochirus splendescens</i>	ATX...TC	C...G	TAG T TAX	XTT	TGTC T TA	T T T C A
<i>Pagurus bernhardus</i>	TTX...TC	C...G	TGG T TAX	XTA	TGTC TGTG	T T T C A
<i>Pagurus acadianus</i>	TTX...TC	C...G	TGG T TAX	XTT	TGTC TGTG	T T T C A
<i>Pagurus pollicaris</i> (NE)	TTX...GT	C...G	ATAG T TAX	XTA	TGTC T A	T T G AT A
<i>Pagurus pollicaris</i> (GU)	TTX...GT	C...G	ATAG T TAX	XTA	TGTC T A	T T G AT A
<i>Pagurus longicarpus</i> (NE)	TTX...GT	C...G	TAG T TAX	XTA G	TGTC TG A	T T AT A
<i>Pagurus longicarpus</i> (GU)	TTX...GT	C...G	TAG T TAX	XTA G	TGTC TG A	T T AT A
<i>Artemia salina</i>	CAATAAATGA	CCAXXXXXXX	TTGATCCTXT	AGATGAATXX	AAAGACCAAG	TTACCTTAGG
<i>Clibanarius vittatus</i>	TGTGTXT...T	TAGGAGTAGT	A...T	TT GAG TT	...TT	...T
<i>Coenobita</i> sp.	A ATXT XT	TG AAATAAA	...	CT AAG TA	...T	...T
<i>Lithodes aequispina</i>	T...C T C	TT ATATAAA	...	A AG TA	...TT	A T
<i>Paralithodes camtschatica</i>	T...C T C	TT ATACAAA	...	A AG TA	...TT	A T
<i>Ellasochirus tenuimanus</i>	T...T T T	TT ATA AAA	...	GA AG TA	...TT	A T
<i>Labidochirus splendescens</i>	T...TGT	TT ATA AAA	...	A AG TA	...TT	A T
<i>Pagurus bernhardus</i>	T...T T T	TT GTA AAA	...	A AG TA	...TT	A T
<i>Pagurus acadianus</i>	T...T T T	TT ATA AAA	...	A AG TA	...TT	A T
<i>Pagurus pollicaris</i> (NE)	TG...TGT	TT ATG TGC	...	TA GAG TA	G...TT	...T
<i>Pagurus pollicaris</i> (GU)	T G TGT	TT ATA TAG	...	TA AAG TA	G...TT	...T
<i>Pagurus longicarpus</i> (NE)	T...GT	TT TTA ATA	...	TA AG TA	...TT	A T
<i>Pagurus longicarpus</i> (GU)	T...GT	TT TTA ATA	...	TA AG TA	...TT	A T
<i>Artemia salina</i>	GATAACAGCG	TAATTCTTTT	TTGAGATTTC	AAATCGACAA	AAGAGTTTGC	GAGCGCTGATG
<i>Clibanarius vittatus</i>	...T X	...	C...TT	TT AG	A A	X
<i>Coenobita</i> sp.	C X...C	...	CC...A G	A A	X	...
<i>Lithodes aequispina</i>	...T X	...	...	...	...	...
<i>Paralithodes camtschatica</i>	...T X	...	...	...	...	...
<i>Ellasochirus tenuimanus</i>	...T X	...	...	...	...	...
<i>Labidochirus splendescens</i>	...T X	...	...	...	...	...
<i>Pagurus bernhardus</i>	...T X	...	...	...	...	...
<i>Pagurus acadianus</i>	...T X	...	...	...	...	...
<i>Pagurus pollicaris</i> (NE)	...T X	C	...	...	...	...
<i>Pagurus pollicaris</i> (GU)	...T X	C	...	...	...	...
<i>Pagurus longicarpus</i> (NE)	...T X	C	...	...	...	...
<i>Pagurus longicarpus</i> (GU)	...T X	C	...	...	...	...

Sequences were aligned using CLUSTAL¹⁹, with gaps given a weight of ten and transitions unweighted. Gaps are indicated by Xs. NE and GU refer to populations from New England and the Gulf of Mexico, respectively. Species were identified^{11,13,20} and adult tissue was homogenized in buffer (400 mM EDTA, 10 mM Tris-HCl, 2% sodium sarkosyl, pH 9.4), extracted with phenol/chloroform/isoamyl alcohol (25:24:1), and precipitated with isopropyl alcohol and 1/10 volume of 4.4 M ammonium acetate. DNA was resuspended in water, and further purified by the GENE CLEAN (BIO 101) procedure. Fragments ~529 base pairs long (size based on *Artemia salina* sequence; GENBANK: Shmrtrgda.Or) were amplified by the polymerase chain reaction (PCR)²¹ from whole genomic DNA using primers 16sar (CGCCTGTTAT-CAAAAACAT) and 16bsr (CCGGTCTGAATCAGATCAGT)²² for 40 cycles in a Perkin Elmer Cetus machine (at 94 °C for 1 min; 50 °C, 1.5 min; 72 °C, 2.5 min). Direct DNA sequencing of double-stranded PCR products²² was carried out by dideoxy chain termination²³ using T7 DNA polymerase (Sequenase, version 2.0; US Biochemical) and radioactive labelling with S35 (Amersham).

Fig. 2). These relatively narrow confidence limits are especially remarkable considering the small size of the DNA fragment¹⁴.

The loss of the shell-living habit in hermit crab lineages and the acquisition of a crab-like form (carcinization) may have occurred by heterochrony, an evolutionary change in the timing of development. The ontogeny of *Birgus latro*, a close relative of *Coenobita*⁴ (Fig. 1), illustrates this process. This crab has undergone carcinization independently of the king crab lineage. As a juvenile, *Birgus* lives in a gastropod shell like any hermit crab, but as its ontogeny progresses, it literally outgrows the shell-living habit. Its carcinized adult stage has a calified abdomen and does not carry a gastropod shell¹⁵. This extension of the ancestral hermit crab ontogeny to produce a carcinized adult can be termed peramorphosis¹⁶. Two lines of evidence suggest that peramorphosis may also have been responsible for the independent carcinization of king crabs from pagurid ancestors. In two species of *Pagurus*, northern populations of both species show increased adult size and reduced shell-living as compared with southern populations of conspecifics^{6,17}. As with *Birgus*, these species display a correlation between reduction in shell-living and larger size, possibly from a minor extension of their ontogeny. Finally, details of the limb allometries of carcinized hermit crabs, including *Labidochirus* and king crabs (Fig. 1), suggest that the ancestral hermit crab allometries were modified to accommodate an extended ontogeny and larger body size¹⁸.

Although the close relationship of king and hermit crabs is a striking example of a major morphological transformation, the larvae of the two groups are similar^{8,9}. Heterochronic modifications do not appear until metamorphosis⁸, an observation consistent with a hypothesis of peramorphosis. Studies of the molecular and cellular basis of heterochrony in king

crabs should therefore focus on changes that occur during metamorphosis. □

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Movement selection in advance of action in the superior colliculus

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THE primate superior colliculus contains a map of saccadic eye movements^{1,2}. Saccades are high-velocity eye movements to selected targets in the visual field, but little is known about the neural mechanisms responsible for target selection or the related problem of choosing a particular movement from the oculomotor repertoire. Two classes of neurons have been described in the superior colliculus which show bursts of activity before the saccade: discrete bursters display a vigorous pre-saccadic burst and prelude bursters³ show low-frequency activity as a prelude to burst onset. We have designed experiments to test whether prelude activity is related to saccade selection. Our tasks use a cue to specify which of two physically identical visual stimuli is the goal of an impending saccade. This cue is spatially and temporally isolated from the potential targets as well as from visual cues signalling movement initiation. Our results show that prelude activity occurs shortly after information is available for correct saccade selection and, more importantly, the activity is predictive of saccade choice. The results thus suggest that the superior colliculus participates in the process of saccade selection.

Only two of our seven trial types are described. In trial type one (Fig. 1a), the animal was required to maintain fixation of a light-emitting diode (LED) illuminated red or green for 300 to 600 ms. Then, two additional eccentric yellow LEDs were illuminated (one above and one below the horizontal meridian) for 1,000 to 7,000 ms while fixation was maintained. When all three LEDs were extinguished, a saccade to the location of one

of the eccentric targets within 300 ms produced a reward. A red fixation stimulus indicated that the upper LED (or T1) served as a rewarded target and the lower LED (T2) served as a distractor. Conversely, a green fixation stimulus signalled that T2 was the target and T1 was the distractor. (Ottes *et al.*^{4,5} used a similar task for different purposes.) The precise target locations and the colour of the fixation target were varied randomly from trial to trial. For this reason, the (to be) rewarded movement could not be determined until all three LEDs had been illuminated.

During recording sessions, we first plotted the response field of an isolated neuron. Next, two conditions in which an LED was centred in the unit's response field were compared: trials in which that LED served as a target or as a distractor. Because the trials being compared differed only in the colour of the fixation LED (which was always outside the unit's response field), differences in activity occurring before the movement initiation cue can be attributed specifically to whether or not the stimulus was selected as a saccadic goal.

Figure 2a depicts the activity of a prelude burster on a single type one trial. As determined on single target trials, the neuron responded vigorously before movements to the upper target but was silent before movements to the lower target (Fig. 1a). On this trial, the red fixation stimulus signalled that an upward movement would be rewarded. The unit began to discharge shortly after the metrics of the required movement were specified by target onset, 5.5 s before the cue to initiate the saccade.

As illustrated in Fig. 2b, this cell was relatively silent throughout trials in which a green fixation LED signalled that a movement to T2 would be rewarded. The animal responded correctly, as predicted by the prelude activity.

Figure 2c depicts a trial in which the animal made a rare error. As in Fig. 2b, the green fixation light signalled that a movement to T2 would be rewarded. The prelude burster, however, fired vigorously and the animal looked incorrectly to

T1. The metrics of the upcoming saccade, not the colour of the fixation LED, were accurately predicted by the activity of the prelude burster. This pattern of results has been observed in all 22 prelude bursters studied.

We plotted the response fields of prelude bursters to determine how completely these neurons discriminate between targets and distractors. In this analysis, the location of one potential target (for example T2) was fixed where it elicited no response; the other (T1) was moved randomly throughout the response field. On a given trial T1 was either the distractor or the target, permitting us to map the unit's target-movement fields and distractor non-movement fields. Target-movement fields are plots of the horizontal and vertical amplitude of a rewarded movement against either the burst or prelude activity of the unit. Distractor non-movement fields are plots of the x and y position

of the distractor against burst or prelude activity. As shown in Fig. 3a, prelude activity decreases as the selected movement deviates from the unit's best movement. Figure 3c is a target-movement burst field for this same unit. Note that whereas burst rate is double or triple that of the prelude, the spatial tuning of the response is quite similar.

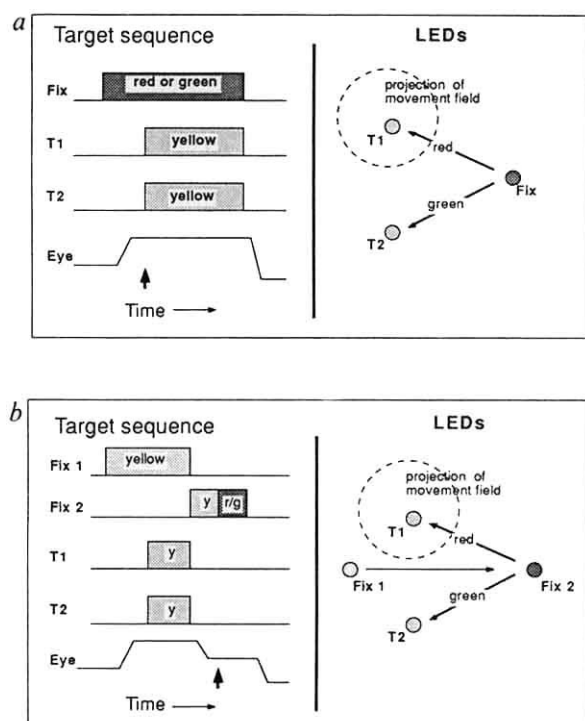


FIG. 1 Trial types used to separate the effects of movement selection and visual stimulation on prelude burster activity. *a*, Trial type 1. Animals first fixated a central red or green LED. After 500 ms, two yellow LEDs were illuminated at eccentric locations. The colour of the fixation LED signalled which of these would be the end-point of a rewarded saccade (the target) and which served as a distractor. After a variable interval of between 1,000 and 7,000 ms, the potential targets and the fixation LED were extinguished and the animal made a saccade to the target's location. This temporally isolated target specification from response initiation. Arrow, onset of the potential targets; this was the point in time at which the animal had sufficient information to select the correct response. *b*, Trial type 2. This identified the coordinate framework used by the prelude bursters. A fixation LED was illuminated yellow (300 to 600 ms); the two potential targets (yellow) were then presented for between 1,000 and 1,500 ms and all three LEDs were extinguished. The animal then made a saccade to a second yellow fixation LED. After 300 to 500 ms this second fixation LED turned red or green, specifying which remembered potential target would serve as the target and which was the distractor. After between 700 and 2,000 ms, the fixation LED was extinguished, releasing the movement. At the beginning of this final interval the animal has sufficient information to select accurately a response. The rewarded saccade to the remembered target is not identical to the retinal eccentricity of the visual target because a saccade has intervened. Activity during this final interval indicates that the response being represented in the prelude burster is independent of the retinal locus of visual stimulation. The arrow indicates the time at which the second fixation LED changed colour, providing the animal with sufficient information to select the correct response.

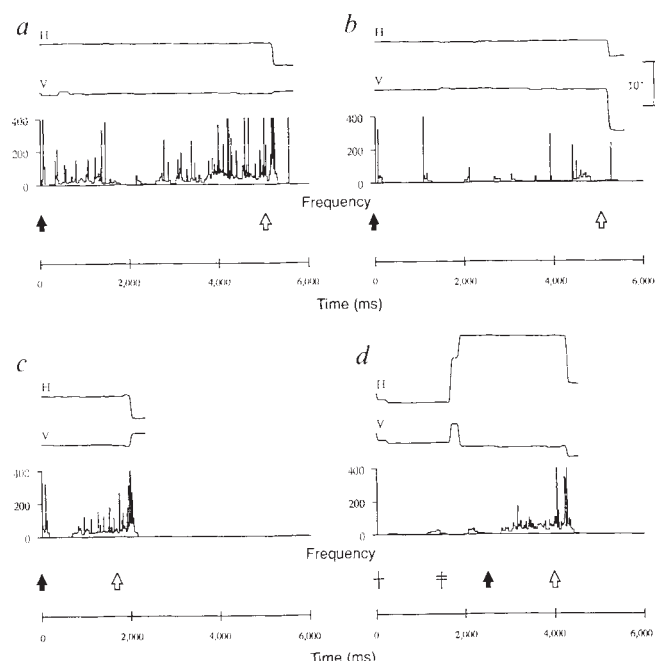


FIG. 2 We used standard electrophysiological methods¹¹ for recording single unit activity and monitoring eye position^{12,13}. Two monkeys (*Macaca mulatta*) were placed on a weekly regimen of water restriction and were trained to perform oculomotor tasks for a water reward. Correct eye movements were reinforced on a variable ratio schedule (VR 2); a 750-ms noise burst served as a secondary reinforcer on all correct trials. Experimental sessions lasted for 2–3 h under dim illumination. Recordings were performed in three colliculi. Four single trial records demonstrate the behaviour of prelude bursters during trial types 1 and 2. For each trial, H and V plot horizontal and vertical eye position respectively. Frequency plots the instantaneous frequency (reciprocal of interspike interval) for a single unit on a single trial. The filled arrow indicates, for each trial type, the time at which sufficient information is available to the animal for correct response selection. *a*, The behaviour of unit rh515a during a type 1 trial. Two targets were presented: one placed to specify a movement to $(-6, 0)$, in the centre of the unit's target-movement field (MF), and the other at $(-6, -14)$, outside the unit's MF. The red fixation LED signalled that a movement to $(-6, 0)$ would be rewarded. The unit became active and stayed active for 5.5 s, until movement onset. The open arrow indicates the offset of all LEDs, cueing movement initiation. The movement that occurred was correctly directed to $(-6, 0)$. *b*, This trial differed from *a* only in the colour of the fixation LED. Here a green LED signalled that a movement out of rh515a's MF would be rewarded. The unit was relatively silent during the 5.5-s interval preceding the movement to $(-6, -12)$. *c*, On this trial the fixation light (green) signalled that a movement to $(-6, -12)$ would again be rewarded. On this occasion unit rh515a became active and produced a burst of activity, accurately predicting an erroneous (unrewarded) movement to $(-6, 0)$. *d*, A type-two trial is presented for unit rh515b (adjacent to rh515a). Firing accurately encoded the impending movement to a remembered target at $(-6, -4)$ only after enough information was available to specify the rewarded movement (filled arrow). Note that information about which stimulus serves as the target and which serves as the distractor is provided by a change in the colour of the second fixation LED. This occurs after both potential targets have been extinguished and an intervening eye movement has occurred. Cross, onset of the potential targets; double cross, time at which both potential targets were extinguished. (Although this unit showed no decrement in response amplitude to remembered targets, for other cells, prelude activity to remembered targets was often reduced when compared with prelude activity to visual targets. The level of reduction varies tremendously from unit to unit; some respond as vigorously to remembered targets as to visual ones, others are nearly inactive before movements to remembered targets.)

How completely does the response of a prelude burster select between a target and distractor? Figure 3*b* plots the prelude activity as a function of the location of the distractor. Figure 3*d* plots the peri-movement burst against the location of the distractor (*x* and *y* coordinates of the potential target to which a movement was not directed). A comparison of Fig. 3*a* with *b* and Fig. 3*c* with *d* reveals that prelude bursters carry a signal from which the response to the distractor is largely, but not entirely, filtered.

Prelude bursters respond differentially to distractors and targets presented at the same location in their response field. A ratio of prelude activity observed with targets at the centre of the target-movement field, with that observed when the same LED serves as the distractor, provides an index of the unit's target/distractor selectivity. Figure 4 is a histogram of this selectivity ratio ($N = 22$). Similar selectivity occurs during the burst period, as can be seen in Fig 3*c* and *d*.

In a second trial type (Fig. 1*b*), after animals fixated a yellow LED for 300 to 600 ms, two yellow potential target LEDs were illuminated simultaneously for 1,000 to 1,500 ms. All three yellow LEDs were extinguished and a second yellow fixation light was illuminated at a new location. After fixation of this second

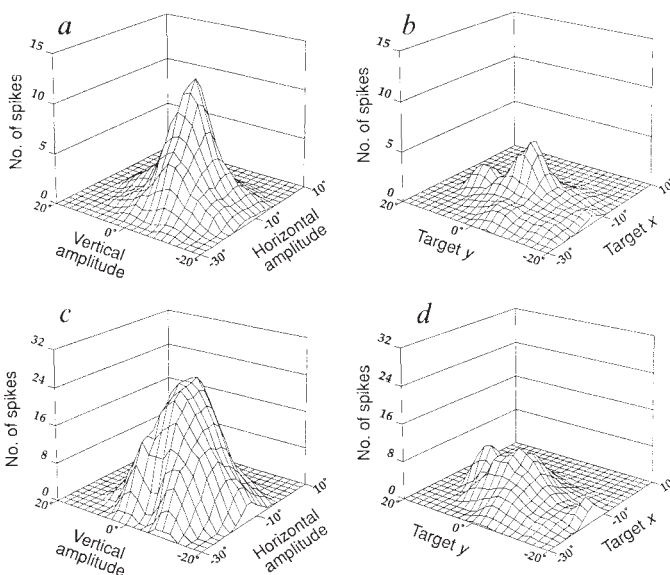


FIG. 3 Four hundred movement trials were used to construct these plots of unit rh0611's target-movement and distractor non-movement sensitivities. *a*, Target-movement prelude field. For each of the 400 trials the number of spikes in the 200 ms before the movement initiation cue is plotted against the horizontal and vertical amplitude of the movement. Prelude activity occurring well in advance of the movement is spatially tuned. *b*, Distractor non-movement prelude field. Here the same unit activity for each of the 400 trials is plotted against the *x*, *y* coordinates of the distractor. *c*, Target-movement burst field. This target-movement field plots the number of spikes occurring from 100 ms before to 100 ms after movement onset against the horizontal and vertical amplitude of the movement. *d*, The same peri-movement activity measured in *d* is plotted against the *x*, *y* coordinates of the distractor. (Note the differences between the ordinate and abscissa pairs on the left and right panels of Fig. 3. The left panels plot actual movements against spike counts. All movements terminated within 3 degrees of the target and were rewarded.) A target-movement field is defined as a plot of the number of spikes in the burst or prelude interval as a function of the horizontal and vertical components of a movement directed towards the (rewarded) target. The term 'target-movement field' is used to identify movement fields constructed of the rewarded movements to one of two potential targets. This terminology is intended to differentiate these rewarded movements, or target movements, from erroneous movements to the distractor. Conventional movement fields use a single target to which all accurate movements are directed.

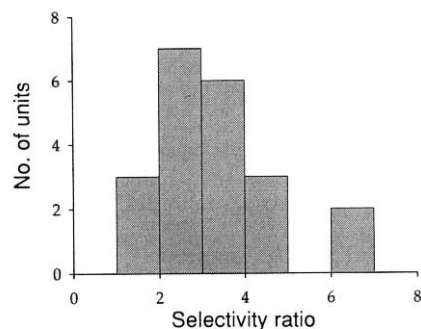


FIG. 4 Prelude selectivity ratio for each unit examined. This measure quantifies the ability of a unit to reject distractors and represent only the selected response. Selectivity ratios were computed from the spike counts of type 1 trials in which one LED was outside the target-movement field of the unit (as determined on single target trials) and the other LED was near the centre of the unit's target-movement field. These rewarded type 1 trials were separated into those directed into the response field of the unit (target trials), and those directed out of the response field of the unit (distractor trials). For each unit, average spike counts on target trials were divided by average spike counts on distractor trials to compute the selectivity ratios. The modal unit showed a selectivity ratio of 3.14:1. Twenty-one units are plotted here. An additional unit with a selectivity ratio of 30 is not plotted.

fixation LED for 300 to 500 ms, the colour changed to red or green, specifying one of the (now absent) potential targets as the saccadic goal. After 500 to 2,500 ms this LED was extinguished. Reward was contingent upon a saccade to the remembered target location within 500 ms. This task allowed us to determine whether the response field of a prelude burster was referenced to the retinal location of the target stimulus or to the metrics of the movement required to fixate the target. This was accomplished by requiring the additional eye movement that intervened between the retinal presentation and the movement to the target.

Figure 2*d* plots a single type-two trial. Prelude activity occurs after the change in colour of the second fixation LED specifies which movement will be rewarded. As with type-one trials, prelude onset is tightly coupled with information about the metrics of the selected movement. On type-two trials, however, that information is not available until after the intervening saccade, at which time the second fixation stimulus changes to red or green.

Examining prelude bursters during these tasks yielded several novel observations. The first is that prelude onset can precede burst onset by at least 7 s. Second, this early responding occurs only after sufficient information is available to permit accurate response selection. Finally, prelude bursters respond differentially to targets and distractors, even if they are identical stimuli. These data are consistent with the hypothesis that the low-frequency activity of the prelude burst cells reflects the output of the covert process of response selection.

Previous studies have identified neurons in the superior colliculus^{6,7}, and other brain areas⁸⁻¹⁰, that become active long before movement onset. It is not known if the activity of these cells qualifies them for identification as target or response selection output elements. Response selection output elements must be differentially sensitive to target and distractor stimuli and changes in their activity must be linked to the time at which sufficient information is available for response selection, as is the case for prelude bursters. Neural signals related to the metrics of a movement, or to the onset of potential targets, are readily observed, but, isolating neural signals related to the process of movement selection is difficult because selection occurs without a behavioral manifestation. Tasks like those in this experiment, which temporally and spatially isolate selection cues, should facilitate an analysis of response selection at the neuronal level. □

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Expansion of an unstable DNA region and phenotypic variation in myotonic dystrophy

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MYOTONIC dystrophy is the commonest adult form of muscular dystrophy, with an estimated incidence of 1 per 7,500, although this is likely to be an underestimate because of the difficulty of detecting minimally affected individuals. It is a multisystem autosomal dominant disorder of unknown biochemical basis¹. No case of new mutation has been proven. We have isolated a human genomic clone that detects novel restriction fragments specific to individuals with myotonic dystrophy. A two-allele *EcoRI* polymorphism is seen in normal individuals, but in most affected individuals one of the normal alleles is replaced by a larger fragment, which varies in length both between unrelated affected individuals and within families. The unstable nature of this region may explain the characteristic variation in severity and age at onset of the disease. A second polymorphism at this locus is in almost complete linkage disequilibrium with myotonic dystrophy, strongly supporting our earlier results which indicated that most cases are descended from one original mutation².

Myotonic dystrophy (DM) is characterized by myotonia and progressive muscle weakness and wasting, with age at onset and degree of severity both being highly variable¹. The disease can be divided into three clinical groups: minimally affected or late onset, classical adult onset, and congenitally affected offspring of affected mothers³. The phenomenon of anticipation, earlier age at onset and increasing severity of disease in successive generations has been ascribed to ascertainment bias⁴ but reassessment of pedigree data⁵ suggests a true biological basis. Cytogenetically detectable rearrangements, which have assisted the cloning of several disease genes⁶⁻¹¹, have not been detected in DM and the nature of the gene remains unknown.

The DM gene has been mapped to a 200-kilobase (kb) interval at chromosome 19q13.3 (refs 2, 12-17). Construction of a radiation-reduced hybrid, 2F5, containing 2 megabases of human chromosome 19, including the closest flanking markers (J.D.B. *et al.*, manuscript submitted), facilitated cloning of this entire region in phage λ . Clones were further localized using radiation-reduced hybrids and pulsed-field gel electrophoresis. Clone λ M9C showed strong interspecies conservation, and a subclone (pBB0.7) identified restriction-fragment-length polymorphisms with *EcoRV* and *EcoRI*. The *EcoRV* polymorphism showed

almost complete disequilibrium with DM (Table 1), much stronger than that reported with *D19S63* (ref. 2), the marker defining the proximal boundary of the candidate region¹⁴. A series of other markers from within this 200-kb DM region failed to detect such strong disequilibrium (our unpublished data). The *EcoRI* polymorphism was detected in normal individuals as fragments of 9 and 10 kb. In 28 out of 34 unrelated affected individuals, we found novel fragments larger than 10 kb (Fig. 1) that were disease-specific (Table 2). Larger fragments were not apparent in the remaining six individuals, but their affected offspring have a larger fragment, suggesting that the novel fragment in the parent was indistinguishable from the normal 10-kb band. The largest fragment so far detected is 15 kb, an increase of 5 kb (Fig. 1a). We have not seen any offspring with a band smaller than that of its parent, although size changes are not always detectable. Enlargement of the variable band has been seen in transmission by either sex and some of the novel fragments appear as smears of variable size (Fig. 1a). No fragments have been observed in the 9-10-kb size range, suggesting that the original mutation in this population occurred on a chromosome lacking the polymorphic *EcoRI* site. In a pedigree showing

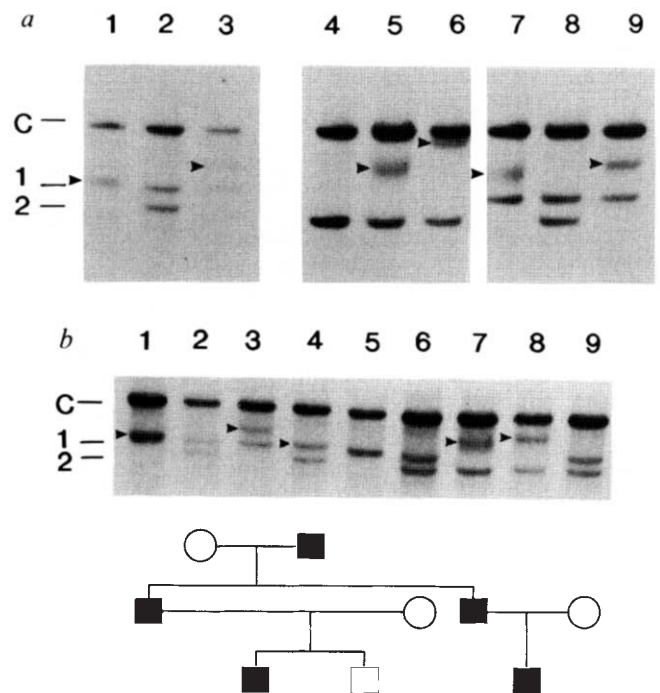
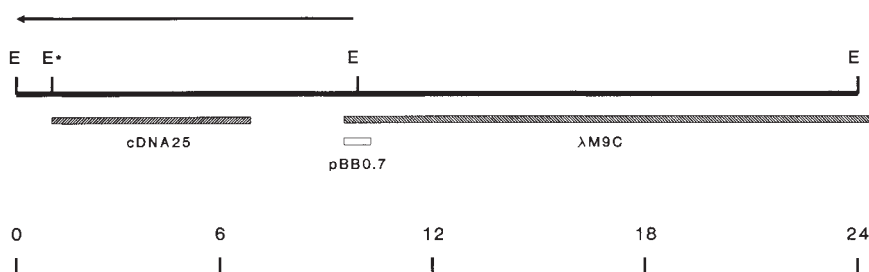


FIG. 1 *EcoRI*-digested genomic DNA probed with pBB0.7, a single copy subclone of λ M9C. All individuals have a constant ~15-kb band (C). Normal individuals are either homozygous or heterozygous for bands of 10 and 9 kb (alleles 1 and 2). Affected individuals have one of these two bands plus a second band of >10 kb (arrowed). a, Lanes 2, 4 and 8 are normal, unrelated individuals; lanes 1, 5 and 7 are unrelated affected individuals; lanes 3, 6 and 9 are affected offspring of individuals 1 and 2, 4, and 5, 7 and 8 respectively. Lane 1 shows one of the smallest size changes detectable (two distinct bands can be clearly seen on the autoradiograph), and lane 6 one of the largest. Lanes 5 and 7 illustrate the smearing of bands seen in some individuals. b, Individual 4 is classified as late-onset and has a novel fragment minimally larger than the normal 10-kb band. His two affected offspring are classified as adult-onset (individual 1 has a minimally increased fragment) and early adult-onset (individual 7 with a novel fragment ~1 kb larger than his father's). The affected grandchildren (individuals 3 and 8) are both classified as early onset and can be seen to have a much larger fragment than their respective parents and their grandparent. Individual 8 had the earliest age at onset and is the most severely affected and also has the largest fragment in this family. $\square$, unaffected; $\circ$, unaffected females.

METHODS. Gel electrophoresis, Southern blotting, probing and autoradiography were done by standard procedures²³.

FIG. 2 *Eco*RI map of the immediate region of pBB0.7 and cDNA25. The long arrow defines the possible location of the region enlarged in DM. E represents an *Eco*RI site, E* represents the polymorphic site. Hybridization analysis of overlapping cloned sequences established the relationship of cDNA25 (ref. 21), λ M9C and the unstable region. cDNA25 detects the same *Eco*RI polymorphism and DM-specific variation as pBB0.7, but not the constant 15-kb fragment; nor does it hybridize to λ M9C or to other clones extending towards the polymorphic *Eco*RI site.



anticipation (Fig. 1b), fragment size increases in successive generations, correlating with increased severity and earlier onset of the disease.

Our findings are remarkably similar to those reported for *FMR* (refs 9–11, 18), where expansion in an unstable region of DNA, a (CGG)_n repeat in exon 1 of the normal *FMR-1* gene, has profound phenotypic effects. It has been proposed that gradual expansion of the repeat ultimately results in the *FMR* phenotype, making it possible that any individual chromosome in the normal population could develop the mutation. A high degree of linkage disequilibrium is observed in normal families between two markers closely flanking the (CGG)_n repeat²⁴. Testing of these markers in fragile X families may reveal a limited number of founding chromosomes. Our results suggest a single origin for most cases of DM, most probably a small (<0.2 kb) insertion or duplication event with an inherent ability to expand. In affected individuals with disease-specific fragments of up to 11 kb, no smearing of autoradiograph bands is apparent. Fragments larger than 11 kb may subsequently undergo somatic expansion, thus explaining the smears seen for

some individuals (Fig. 1a). Expansion occurs whether transmitted by the mother or father; in *FMR*, expansion is usually seen in female transmission, whereas no change or slight contraction is usually seen in male transmission.

Recombination is unlikely to be the mechanism of meiotic expansion in DM because flanking markers would also recombine. In fact few recombinant chromosomes have been detected and strong disequilibrium is observed. This is supported by the observations in *FMR* that markers flanking the (CGG)_n repeat do not recombine and that expansion or contraction of the unstable region can occur in male transmission of a chromosomal region not paired in male meiosis¹⁸.

Expansion of a trinucleotide repeat may prove to be a common mechanism in human genetic disease and has now been observed both in fragile X syndrome (refs 9–11, 18) and Kennedy's disease¹⁹. Increased size of a (CAG)_n repeat in the coding region of the androgen receptor gene is absolutely associated with Kennedy's disease¹⁹, but does not show the same degree of instability associated with *FMR*. The inheritance of an unstable DNA sequence has now been found in DM, although its precise nature is not known (Fig. 2), nor is it apparent whether a trinucleotide repeat is the basis of the expansion in DM, or if it occurs within the coding region of a gene (as in fragile X syndrome and Kennedy's disease) or is located in an intron or an intragenic region. Although our findings identify a potential biological mechanism for anticipation^{5,20}, they do not yet offer an explanation for the maternally transmitted congenital form of the disease. Both λ M9C (this work) and cDNA25 (ref. 21) detect expressed sequences, which we are investigating further. □

TABLE 1 Disequilibrium data for *Eco*RV polymorphism detected by pBB0.7

Allele	Number of DM chromosomes	Frequency	Number of normal chromosomes	Frequency	χ^2	ϕ
1	74	0.99	140	0.60	39.4	0.96
2	1	0.01	92	0.40		

The families in this study are of European origin and have been described previously^{2,17}. Chi-squared (χ^2) values were calculated using standard formulae. The Yule's coefficient of association²² (ϕ) gives an estimate of association which is independent of sample size. Values are between 0 (random association) and 1 (complete association). Results were significant at values of $P < 0.001$.

TABLE 2 Association of the variable *Eco*RI fragment with DM

Genotype	Number of DM individuals	Number of normal individuals
1	0	7
2	1	13
3	5	14
4	12	0
5	16	0
Total	34	34

Genotypes are defined by the fragment sizes detected for each individual: 1 (9-kb fragment), 2 (10-kb fragment), 3 (9+10-kb heterozygote), 4 (9+fragment larger than 10 kb), 5 (10+fragment larger than 10 kb). None of the individuals lacked a normal allele. The degree of association of the variable-size fragment with DM was determined from analysis of the genotype of one affected and one unaffected unrelated individual from each pedigree. A standard χ^2 analysis, with 4 d.f., was carried out on the data. The degree of association of the variable fragment with DM was significant for values of $P < 0.48 \times 10^{-9}$.

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Detection of an unstable fragment of DNA specific to individuals with myotonic dystrophy

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MYOTONIC dystrophy (DM) is the most common form of adult muscular dystrophy, with a prevalence of 2–14 per 100,000 individuals¹. The disease is characterized by progressive muscle weakness and sustained muscle contraction, often with a wide range of accompanying symptoms. The age at onset and severity of the disease show extreme variation, both within and between families. Despite its clinical variability, this dominant condition segregates as a single locus at chromosome 19q13.3 in every population studied¹. It is flanked by the tightly linked genetic markers *ERCC1* proximally^{2,3} and *D19S51* distally^{4,5}; these define the DM critical region. We report the isolation of an expressed sequence from this region which detects a DNA fragment that is larger in affected individuals than in normal siblings or unaffected controls. The size of this fragment varies between affected siblings, and increases in size through generations in parallel with increasing severity of the disease. We postulate that this unstable DNA sequence is the molecular feature that underlies DM.

The DM locus has been characterized both genetically^{6–8} and physically^{9–11} and recent efforts have concentrated on constructing a long-range restriction map^{12,13} of the critical region. The distance between the genetic markers *ERCC1* and *D19S51* is ~600 kilobases (kb)¹². Several HTF islands¹⁴ have been identified within this region¹², indicating that expressed sequences are present.

Yeast artificial chromosome (YAC) clones have been screened with the flanking markers *ERCC1* and *D19S51*, and a contig around the latter probe has been described (J.B. *et al.*, manuscript submitted). A more comprehensive cosmid and YAC contig has been constructed across the entire DM critical region¹⁵. Because linkage disequilibrium studies suggested that the probe *D19S63* (ref. 16) is closest to the DM locus¹⁷, YACs containing this probe were characterized. These proved to be unstable and prone to extensive deletions and rearrangements¹⁵. Despite this, we have purified the insert from a YAC that contains the markers p36.1, p37.1 and p20.1, which are known to lie within 100 kilobases (kb) of *D19S63* (ref. 12). This was used to screen complementary DNA libraries, a feasible approach for isolating expressed sequences from large tracts of DNA^{18,19}. In this way we identified a 4.5-kb cDNA (termed cDNA25) from a human fetal brain library (Stratagene), which we have shown maps back to the DM critical region using

pulsed-field gel electrophoresis and somatic cell hybrids (data not shown).

The cDNA25 probe detects an *EcoRI* polymorphism (Fig. 1a) with alleles of 9.8 and 8.6 kb, the frequencies of which (calculated from 30 unrelated Caucasians) are 0.53 and 0.47 respectively. When we investigated DNA from unrelated individuals affected with DM, only one of the alleles found in unaffected individuals was present, together with an additional, larger fragment (Fig. 1b). Of 19 unrelated DM patients, this larger *EcoRI* fragment (which varies in size from 9.8 to 14.6 kb) has been detected in 15. But we have not so far detected the larger fragment in any of 30 unrelated, unaffected individuals. The absence of a DM-specific band in 4 of the 19 DM individuals may be the result of a size increase not detected at the resolution of our gels. This finding may be clinically significant in that all the individuals without a DM-specific fragment were mildly affected with the disease at the time of sampling. The size increase is not detectable with all enzymes, indicating that the unstable DNA sequence may not be contained within cDNA25, although the two must be on a single *EcoRI* fragment. This is probably the 9.8-kb allele as no intermediate fragments between the two normal alleles have been detected.

The inheritance of this additional fragment has been studied in DM families, and it always co-segregates with the disease. Moreover, the size of the larger fragment, although variable between siblings, increases in subsequent generations (Fig. 2). In this respect, the situation is similar to that in the fragile X syndrome^{20,21} and in X-linked spinal and bulbar muscular atrophy²². In fragile X and DM cases, the variable band appears either as a single allele, or as a smear of bands of varying intensity. This suggests that although the instability in DM is meiotic, it also shows somatic mosaicism due to mitotic instability, at least in lymphocyte populations. A fragile site maps in the vicinity of the DM gene on 13q13.3. Fragile X results from

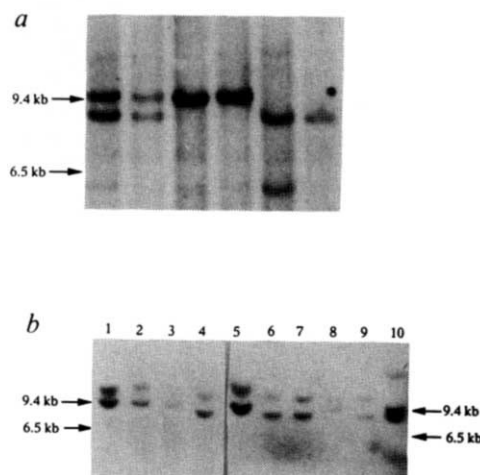


FIG. 1 The genomic fragments detected by cDNA25 in *EcoRI*-digested DNA from normal controls and individuals affected by myotonic dystrophy. *a*, The restriction-fragment-length polymorphism detected in six normal unrelated individuals. Allele sizes are 9.8 and 8.6 kb. Several fainter bands are also present (20, 6.8 and 5.5 kb) owing to the presence of sequences that are highly related to cDNA25. The intensity of these bands varies, both with the amount of DNA in each lane and the duration of hybridization. *b*, The variation seen in the size of the unstable fragment specific to DM patients in 9 unrelated affected individuals (tracks 1–9) compared with the normal allele sizes (track 10). The 20-kb band detected by one of the related sequences mentioned is also visible in track 10.

METHODS. DNA (3 µg) was digested with *EcoRI* and the fragments separated on a 0.6% agarose gel. It was transferred by alkali-blotting onto Hybond N+ (Amersham) and hybridized to ³²P-labelled cDNA25 for 16 h at 65 °C. The filter was washed to a stringency of 0.1 × SSPE (18 mM NaCl, 1 mM sodium phosphate, 0.1 mM EDTA, pH 7.7) and autoradiography was for 2–5 days at –70 °C.

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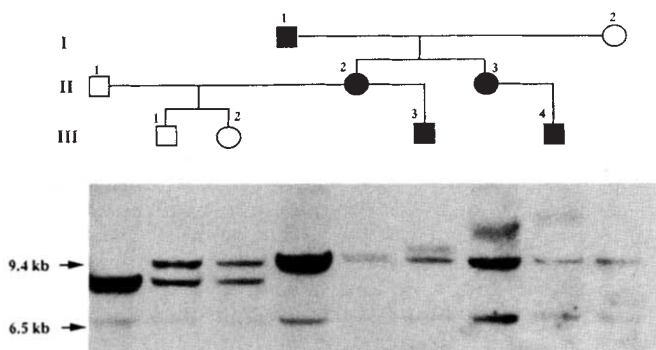


FIG. 2 Association of the variable fragment size and the severity of symptoms seen in a family affected by myotonic dystrophy. *EcoRI*-digested DNA probed with cDNA25 shows the fragment size increasing as it is passed through the generations. It is seen as a discrete band (individuals II2 and III3) or a smear (individuals II3 and III4) and correlates well with the increase in severity of the symptoms in each individual: individual I1 has mild symptoms (cataract); individual II2 has mild symptoms with myotonia detectable by electromyography; individual II3 has classic symptoms of DM; individual III3 has frank myotonia in hands and tongue; individual III4 has severe symptoms and mental retardation.

expression of a fragile site within the *FMR-1* gene²³; a phenotypic effect is seen when the (CGG)_n repeat in the *FMR-1* gene exceeds 52 units in length²⁴. It will be interesting to see if a similar mechanism underlies DM, although no chromosomal abnormalities have ever been associated with this disease¹.

Of particular interest is the finding that the size increase of the DM fragment is correlated with increasing severity of symptoms, illustrated by the family shown in Fig. 2. The phenomenon of anticipation (the increasing severity of the disease in subsequent generations of DM families) has been identified before^{1,25} and is discussed in an accompanying paper in relation to this DNA mutation²⁶. The congenital form of DM is only inherited from the maternal line, the risk of a DM child having this form of the disease being dependent on the severity of the mother's symptoms during pregnancy²⁷. Our initial data suggest that apart from congenital individuals, there are no significant differences in the phenotype when comparing inheritance through the maternal and paternal lines.

Whether the DNA instability detected by cDNA25 is a cause or effect of the complex phenotype seen in DM needs further investigation. In fragile X syndrome the unstable sequence lies within the translated sequence of *FMR-1* (ref. 23), and in X-linked spinal and bulbar atrophy it is in the coding region of the gene for the androgen receptor²². Although our cDNA25 clone might not contain the variable sequence, the mutation may still affect its expression, or that of other genes in the DM critical region. The identification of a DNA sequence effect that is always associated with classical DM symptoms and which correlates with severity should allow better presymptomatic and prenatal diagnosis, as well as permitting new approaches to understanding the molecular pathology of the disorder. □

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Cloning of the essential myotonic dystrophy region and mapping of the putative defect

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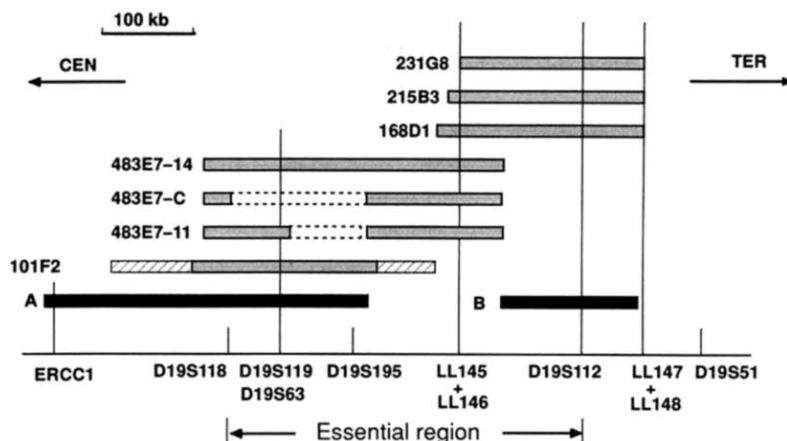
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MYOTONIC dystrophy is a common dominant disorder (global incidence of 1:8,000) with variable onset and a protean nature of symptoms mainly involving progressive muscle wasting, myotonia and cataracts¹. To define the molecular defect, we have cloned the essential region of chromosome 19q13.3, including proximal and distal markers^{2–7} in a 700-kilobase contig formed by overlapping cosmids and yeast artificial chromosomes (YACs). The central part of the contig bridges an area of about 350 kilobases between two new flanking crossover borders^{4,5}. This segment has been extensively characterized through the isolation of five YAC clones and the subsequent subcloning in cosmids from which a detailed *EcoRI*, *HindIII*, *MluI* and *NorI* restriction map has been derived. Two genomic probes and two homologous complementary DNA probes were isolated using the cosmids. These probes are all situated within ~10 kilobases of genomic DNA and detect an unstable genomic segment in myotonic dystrophy patients. The length variation in this segment shows similarities to the instability seen at the fragile X locus⁸. The physical map location and the genetic characteristics of the length polymorphism is compatible with a direct role in the pathogenesis of myotonic dystrophy.

As a prerequisite to the isolation and characterization of the defect causing myotonic dystrophy (DM), we have prepared cosmid contigs by chromosome walking in our chromosome 19-enriched cosmid library⁹ starting from polymorphic markers

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FIG. 1 Physical and genetic map for the DM region of chromosome 19q13.3. The map shows the position of various genetic loci, relative to the position of overlapping YAC clones and a proximal 350-kb (A) and distal 150-kb (B) cosmid contig. The cosmid contigs (solid black bars) were prepared by chromosome walking, starting from the proximal *ERCC1* locus and the distal *D19S112* locus, as described^{4,5}. New polymorphic loci have been derived from the proximal contig: *D19S118* (ref. 12), *D19S119* (ref. 13) and *D19S195* (ref. 21). Marker *D19S63* represents the position of an independently isolated polymorphic marker^{22,23}, revealing the same polymorphism as *D19S119*. The region designated as 'essential' is flanked by the nearest crossover markers. The location of the elusive DM locus is restricted to this essential area. *ERCC1* and *D19S51* were the previously closest flanking markers^{6,7}. YAC clones used here are indicated by grey bars. The cross-hatched regions in one of the YACs indicate uncertainties with respect to the origin of the ends, based on the finding that this YAC (101F2) maps to multiple human chromosomes as scored by *in situ* hybridization (M.V., unpublished results). The dashed areas in two of the YACs (483E7-C and 483E7-11) indicate regions deleted in the highly unstable original clone (manuscript in preparation). The clones 231G8, 215B3 and 168D1 are unstable, showing multiple bands in the range of 180–250 kb. The YACs are designated as for the St Louis YAC library¹⁴,



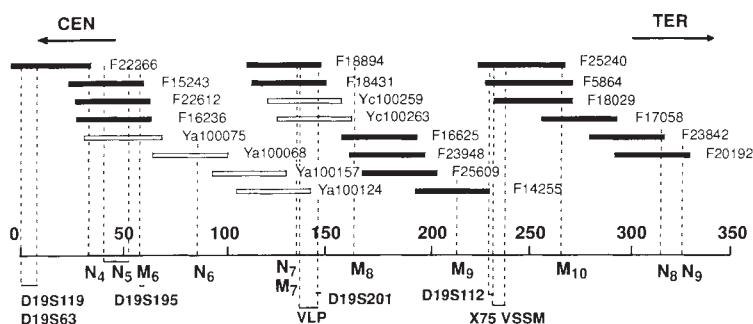
except that plate-numbering is different: 101F2 = A107F2; 168D1 = A51D1; 215B3 = B5B3; 231G8 = A25G8 and 483E7 = B136E7. CEN and TER indicate the centromeric and telomeric directions. PCR primers derived from the vector/insert junctions of YAC 231G8 are indicated as LL145/146 and LL147/148 (manuscript in preparation).

flanking the DM region. We thus established a cosmid contig extending 350 kilobases (kb) towards DM from the proximal flanking marker *ERCC1* (refs 2–4), a small cosmid/YAC contig around the distal marker^{6,10} and a 150-kb cosmid contig around locus *D19S112* (refs 5, 11). Several new polymorphic markers that were informative in DM families were derived from the proximal cosmid contig⁴. These markers have permitted the mapping of a proximal crossover in between *D19S118* (ref. 12) and *D19S119* (ref. 13), hence eliminating about 200 kb from the proximal side of the DM candidate region⁴. A highly informative marker, X75bVSSM, has been developed which lies within a few kb of *D19S112* (ref. 5). This marker is informative in a distal crossover family, so is the nearest distal marker flanking the DM candidate region. On the basis of the orientation of the two flanking cosmid contigs and the positions of the new DM flanking markers, we estimate that about 220 kb of the candidate region is represented in the two contigs established by chromosome walking^{4,5}. Figure 1 shows a map of the DM region, including the location of various markers and contigs.

To characterize the region between the distal and proximal cosmid contigs, we isolated and mapped cosmid clones from the central DM region. We could not extend the contigs by chromosome walking because we were unable to find further

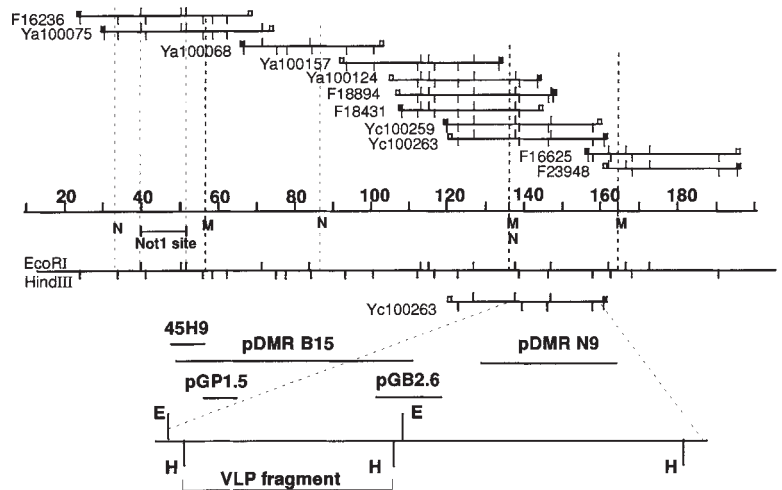
overlapping clones in the eightfold-redundant chromosome 19 cosmid library. We therefore searched a YAC library¹⁴ by screening with polymerase chain reaction (PCR) primers for the X75b VSSM (for *D19S112* (refs 5, 11) and *D19S119* (ref. 13)), which are derived from the distal and proximal cosmid contigs, respectively. Use of the *D19S119* primer pair resulted in the isolation of a 350-kb chimaeric YAC, 101F2, with insert sequences spanning ~200 kb of the proximal cosmid contig (Fig. 1). With the distal X75b VSSM primers, three new YAC clones were isolated. Two of these YACs (215B3, 231G8) were analysed in more detail, revealing both clones to be non-chimaeric and unstable. Using Alu-PCR products from 231G8 to probe the chromosome 19 cosmid library, a small cosmid contig consisting of clones F18894 and F18431 was established in the central gap region (C.A., unpublished data; and Fig. 2). New PCR primers, derived from the vector/insert junctions from 231G8, were used to isolate various new YACs, including the highly unstable 483E7 clone (Fig. 1). This YAC overlaps with the proximal YAC 101F2 and the distal YAC 215B3. The extreme instability of this bridging YAC clone was evident from the many related YAC bands, ranging from 150–250 kb, with only a minor trace of the 320-kb undelated form (not shown). Two stabilized deletion forms (483E7-C, 180 kb, and 483E7-11, 245 kb) and YAC 215B3 were

FIG. 2 Cosmid contig map for the central DM region. Cosmids derived from the chromosome 19 library are shown as black bars and their identification numbers are preceded with 'F'; cosmids obtained by subcloning from YACs are shown as white bars. 'F'-cosmids on the left (proximal contig) or right (distal contig) were originally found by chromosome walking in the chromosome 19 cosmid library, as described^{4,5}. Cosmids between the proximal and distal contigs were found by hybridization with Alu-PCR products from YAC 231G8 (for cosmids F18894 and F18431) or were generated by subcloning from YACs 483E7-C (identification number preceded by Ya) and 231G8 (preceded by Yc). The YACs were subcloned in the Lawrist 13 vector following partial *MboI* digestion of total yeast plus YAC DNA. Clones with human inserts were screened by colony hybridization with human repetitive DNA probes using standard techniques. Cosmid overlaps were established using chromosome walking as before^{4,5} and by fluorescent fingerprinting of cosmid DNAs^{15,16}. Overlaps were also confirmed by the construction of an *EcoRI* and *HindIII* restriction map for the entire contig. Only the central part of this restriction map is depicted (Fig. 3). The restriction map was used to locate sites for two rare-cutting restriction enzymes relative to the *EcoRI* and *HindIII* sites. The location of *NotI* and *MluI* sites are indicated along the horizontal bar showing the DNA length in kb from locus *D19S119*; the sites are designated as N or M, with subscripts indicating the identification number of the particular site, starting from the more proximal *ERCC1* locus. Sites with lower subscript numbers have been described as part of the proximal cosmid contig⁴. Note the high frequency of occurrence within the 700 kb contig: 9 *NotI* sites and 10 *MluI* sites. Also indicated are the precise locations, derived from the *EcoRI/HindIII* map, for various loci: *D19S119* (ref. 13), *D19S63* (refs 22, 23), *D19S195* (ref. 21), *D19S201* (ref. 17) and *D19S112* (ref. 11). The location of the *EcoRI* fragment containing the variable length polymorphism is designated by VLP.



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FIG. 3 Restriction map of the central DM region. Restriction sites for *EcoRI* and *HindIII* are indicated along the overlapping cosmids and along a horizontal bar indicating the distance in kb starting from the *D19S119* locus¹³. Amplified portion of the map shows the locations of probes discussed in the text and used to detect the variable length polymorphism (VLP) in DM patients.



subcloned into cosmids for detailed genomic analysis (see Fig. 2 legend). The resulting cosmids were ordered (Fig. 2) by chromosome-walking^{4,5} and by cosmid fingerprinting^{15,16}. To help with the localization of candidate genes, a restriction map for *EcoRI*, *HindIII*, *MluI* and *NotI* sites was made for the central DM area (Figs 2 and 3).

To isolate candidate genes for DM, we searched for cDNAs corresponding to various cosmids from the central DM region. Two distinct cDNA clones (pDMR B15 and pDMR N9) were found in a mouse brain lambdaZAP cDNA library using the overlapping cosmids F18894 and Ya100263 as probes, respectively (G.J., manuscript in preparation). In addition, using a cosmid (Ya100172) largely overlapping with F18894, a 700-base-pair (bp) 5'-truncated cDNA (45H9) was isolated from a human fetal brain cDNA library¹⁷. Comparison of partial 3'-cDNA sequences from pDMR B15 and 45H9 indicates that these cDNAs represent the murine and human forms of the same

gene (G.J., unpublished results). This conclusion is corroborated by the mapping of the two cDNAs to the same 10-kb *EcoRI* fragment in F18894 and 9-kb *EcoRI* fragment in Ya100263 (Fig. 3). The *EcoRI* fragments in these two cosmids have been derived from different individuals and correspond to allelic forms, which have also been detected using pDMR B15 as a probe on genomic *EcoRI* blots from normal individuals (not shown). Surprisingly, pDMR B15 detects a very unusual variable-length polymorphism (VLP) in *EcoRI*- or *KpnI*-digested DNA from DM patients, with allele sizes ranging between 10–15 kb (not shown). This has also been observed using various genomic probes derived from the same segment of cosmid Ya100263 on Southern blots of DNA from DM patients digested with *EcoRI*, *HindIII* or *KpnI*. Probe pGP1.5, containing a 1.5-kb genomic *PstI* fragment (Fig. 3), reveals the identical *EcoRI* variable-length polymorphism (not shown) and also detects a *HindIII* polymorphism in DM patients (Fig. 4). In the normal human population (over 100 individuals analysed), two *HindIII* allele sizes of 8.5 and 9.5 kb have been found using the pGP1.5 probe. In contrast, a survey of ~200 DM-affected individuals reveals increased *HindIII* allele sizes in the range of 9.5 to 15 kb in nearly 70% of the molecularly diagnosed DM individuals. This is illustrated in a three-generation DM family (Fig. 4). Strikingly, the allele sizes appear to increase in successive generations of this DM family, correlating with an increased severity of the disease. The individuals in generation III, which suffer from a severe congenital form of the disease, also have the largest (15 kb) *HindIII* alleles. We are in the process of analysing all our DM families ($n > 200$) to establish whether there is a strict positive correlation between the expanding allele size and the severity of the disease. The same variable-length-polymorphism patterns have been observed for probe pGB2.6, which is slightly more distal than pGP1.5 (Fig. 3), and for cDNA25 (ref. 18). cDNA25 hybridizes to our cosmid F18894 (K.J., unpublished results) and so may be homologous to our cDNAs B15 and 45H9.

Through the use of YAC and cosmid cloning strategies and novel mapping approaches, we have identified a segment of 10-kb DNA containing a highly unstable genomic sequence. By Southern blot analysis, this instability appears to be similar to the genomic events seen in fragile X syndrome^{8,19,20} and in spinal and bulbar muscular atrophy²⁵. The sequence tends to increase in size only in DM-affected individuals and is situated adjacent to a transcribed region. Other transcribed regions are in the immediate vicinity of this gene. Although we have no proof that expressed sequences are directly involved, we now have the tools to study the genetic events that result in the development and transmission of the complex clinical features in myotonic dystrophy. □

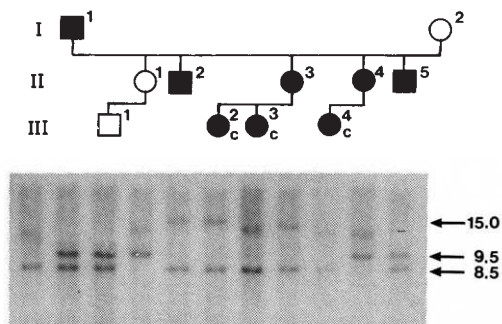


FIG. 4 Pedigree and Southern blot analysis of a three-generation DM family. The pedigree illustrates affected males (black squares) and females (black circles) that show clinical manifestations of DM. The DM-affected individuals in generations I and II show both clinical and electromyographic evidence of myotonia using the criteria defined in ref. 24. Three affected individuals born with the congenital form of the disease (designated by 'c') are shown in generation III. Southern blot analysis with probe pGP1.5 was done on *HindIII*-digested DNA from peripheral blood leukocytes for all individuals. Normal members of the family (open squares and circles) display polymorphic *HindIII* fragments of 8.5 and 9.8 kb. The DM-affected grandfather (individual I-1) shows enlarged *HindIII* bands cosegregating with the DM chromosome. The affected individuals of generation II have all inherited expanded alleles, slightly larger than those seen in individual I-1. All three congenital DM patients of generation III show even larger DM-associated *HindIII* alleles (up to 15.0 kb). Note that the hybridization signals of the expanded alleles appear blurred, implying variability in the size of the enlarged *HindIII* bands and suggesting instability of the DM region with somatic cell heterogeneity.

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Evidence from mosaic analysis of the masculinizing gene *her-1* for cell interactions in *C. elegans* sex determination

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SEX in *Caenorhabditis elegans* is determined by a regulatory cascade of seven interacting autosomal genes controlled by three X-linked genes in response to the X chromosome-to-autosome (X/A) ratio^{1,2}. XX animals (high X/A) develop as self-fertile hermaphrodites, and XO animals (low X/A) develop as males. The activity of the first gene in the sex-determining cascade, *her-1*, is required for male sexual development³. XO *her-1* loss-of-function mutants develop as self-fertile hermaphrodites, whereas XX *her-1* gain-of-function mutants develop as masculinized intersexes⁴. By genetic mosaic analysis using a fused free duplication linking *her-1* to a cell-autonomous marker gene, we show here that *her-1* expression in a sexually dimorphic cell is neither necessary nor sufficient for that cell to adopt a male fate. Our results suggest that *her-1* is expressed in many, possibly all, cells and that its gene product can function non-autonomously through cell interactions to determine male sexual development.

Genetic analysis has indicated that the terminal regulator *tra-1* (Fig. 1a), which controls sexual phenotype in all dimorphic tissues⁵, acts cell-autonomously⁶. But analyses of intersexual animals resulting from intermediate X/A ratios⁷ or *sdc-1* loss-of-function mutations⁸ are consistent with a non-cell-autonomous function for one or more of the genes that regulate *tra-1*. We have analysed *her-1* genetic mosaics to determine whether this gene functions non-cell-autonomously, and if so, which cells

must express *her-1* to cause male development. As *her-1* regulates *tra-1* through at least five intervening genes, the phenotypes of *her-1* genetic mosaics will reflect the functions of these genes as well as *her-1*. If all these genes function cell-autonomously, then in XO animals the sexual fate of each cell will reflect its *her-1* genotype. But if one or more functions non-cell-autonomously, then *her-1*(+) cells may follow hermaphrodite fates, or *her-1*(-) cells, male fates.

To test for cell autonomy, we generated a fused free duplication (*ctDp11*) that carries both *her-1*(+) and the wild-type allele of the normally unlinked *ncl-1* gene (Fig. 1b), which cell-autonomously controls nucleolar size (E. Hedgecock, personal communication). Spontaneous somatic loss of the fusion duplication in an animal chromosomally mutant for these two loci produces *ncl-1 her-1* genetic mosaics. Cells that have lost the duplication can be identified by their enlarged nucleoli (Ncl phenotype). Scoring of relatively few cells for Ncl in a larva or adult allows one to determine from the known cell lineage (Fig. 1c)^{9–11} the precise point of duplication loss and to infer the *her-1* genotype of every cell in most mosaic animals. The possibility of generating such fusion duplications (suggested by R. Herman), which link a gene of interest to a normally unlinked cell-autonomous marker gene, substantially increases the usefulness of genetic mosaic analysis.

Among XO animals carrying the fusion duplication we observed mosaics that displayed two kinds of *her-1* non-autonomy. In cases of the first kind, some or all of the *her-1*(-) cells, expected to follow hermaphrodite fates if *her-1* function were required cell-autonomously, instead followed male fates, indicating that they were masculinized by the remaining *her-1*(+) cells. For example, two mosaics in which the duplication was lost at first cleavage such that all AB-derived cells were *her-1*(-) nevertheless developed as complete males (Table 1, line 1), showing that *her-1* function in only P₁-derived cells can be sufficient for normal male development. This phenotype was variable, however: another such animal (line 2) was intersexual, with some of the *her-1*(-) cells following hermaphrodite fates, and a third died before reaching sexual maturity. Non-autonomous masculinization was also observed in mosaics resulting from later losses in the AB lineage (lines 4–23; Fig. 2a, b), and in the P₂ lineage (lines 27–29, for example). These results indicate that the *her-1* gene product is not cell-autonomously required for male development in any of the sexually dimorphic cells of the AB and P₂ lineages.

In cases of the second kind, *her-1*(+) cells that were expected to follow male fates if *her-1* function were cell-autonomous, instead followed hermaphrodite fates. This indicates that, surprisingly, *her-1*(-) cells can feminize *her-1*(+) cells. For example, two mosaics in which all P₁-derived cells were *her-1*(-) and all AB-derived cells were *her-1*(+), developed as intersexes in which not only all the P₁-derived, but also some of the AB-derived cells formed hermaphrodite structures (Table 1, lines 24, 25; Fig. 2c, d). Further examples of non-autonomous feminization in mosaics resulting from later losses in the P₁ lineage are shown in the lower half of Table 1. Non-autonomous feminization was also observed as the result of duplication losses in AB-derived cells (lines 3, 4, 10, 18, 19). These results indicate that in both AB- and P₁-derived cells, *her-1* expression is not cell-autonomously sufficient for male development.

In six of the animals showing extensive feminization of *her-1*(+) cells, no cells with the Ncl phenotype—indicating *her-1*(-) genotype—could be seen (lines 36–41). The Ncl phenotype cannot be scored in cells of either the intestine (E descendants) or the germ line (P₄ descendants). But the duplication was shown to be present in the germ line of several similar animals by direct visualization of extrachromosomal material in oocytes after staining with DAPI (4',6-diamidino-2-phenylindole). The animals in lines 36–41, therefore, may lack the duplication in the E lineage only, which would indicate a strong feminizing influence of *her-1*(-) intestinal cells on other sexually dimorphic lineages.

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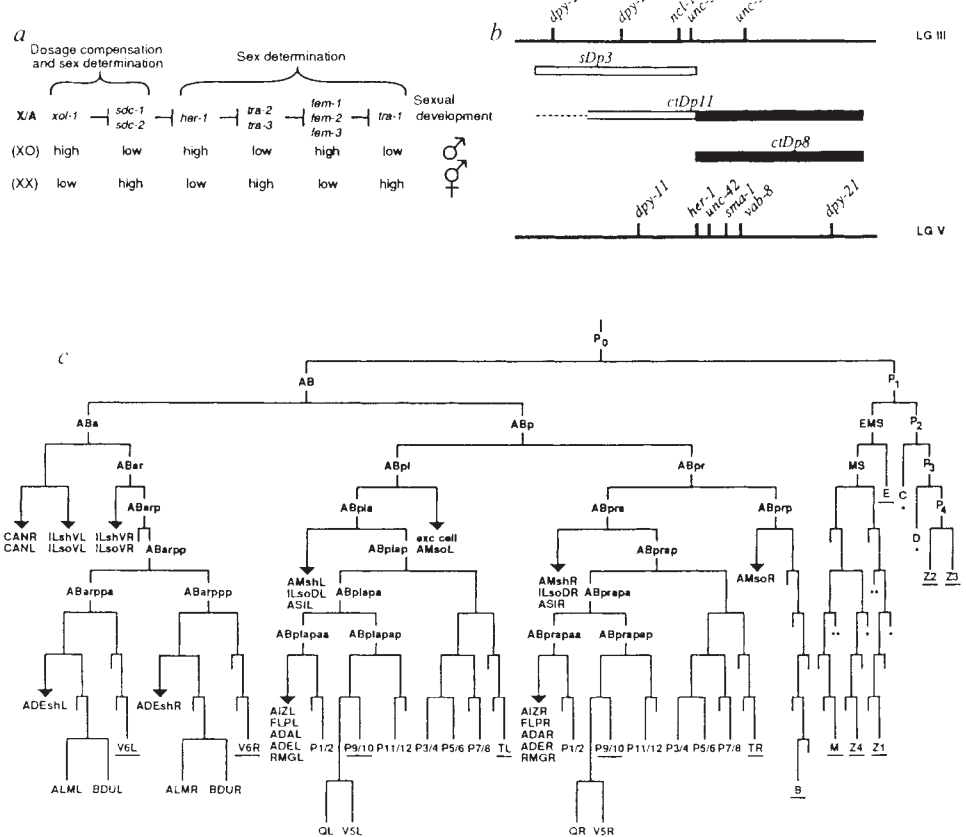
In all of seven animals in which the germ line was presumably *her-1*(-), but the intestine and somatic gonad followed male fates (lines 27-29), only sperm were produced, suggesting that *her-1* expression in cells other than the germ line can inhibit oogenesis. Conversely, in eight of nine animals (lines 34-41) in which the germ line was presumably *her-1*(+), and either the intestine or somatic gonad followed hermaphrodite fates, the germ line produced both sperm and oocytes. The one exception in this class was an animal (line 35) with abnormal gonad morphology, which could have been responsible for the lack of oocytes. These results suggest that the sexual identity of the germ line can be determined not by its own *her-1* genotype, but rather non-autonomously as a result of either the sexual

phenotype or the *her-1* genotype of the gonad and intestine.

Three other general features of the mosaics analysed can be seen from Table 1. First, all but one showed some non-autonomy of *her-1* function, including those that did not survive to adulthood. The exceptional animal apparently lacked the duplication only in the non-sexually dimorphic C lineage (line 38). Second, mosaic phenotypes were variable: when two or more animals with duplication losses at the same point in the cell lineage were analysed, they generally showed similar but non-identical patterns of intersexual development. Third, mosaics with duplication losses in the AB lineage showed predominantly masculinization of the resulting *her-1*(-) cells by the remaining *her-1*(+) cells, whereas mosaics with losses in the P₁ lineage showed

FIG. 1 *a*, Genetic control of sex determination in *C. elegans* (modified from refs 1, 2). *b*, Origin and structure of the fused free duplication *ctDp11*. Diagram shows genes on LGIII and LGV included in the free duplications *sDp3*¹⁸ and *ctDp8*, respectively, as well as the structure of the fusion duplication (orientation of the two fragments was not determined), which includes at least the indicated genes. Map positions of marker genes are from ref. 19. *c*, Lineal relationships¹¹ of cells scored for sexual and Ncl phenotypes in XO *her-1* mosaics. Cells are positioned (anterior daughters to the left) and named conventionally according to ancestry or, for specific differentiated cells, according to function¹¹. Arrows indicate one or more additional cell divisions. Sexually dimorphic blast cells are underlined. These cells, other named cells at the ends of lineage branches, and cells that give rise to body wall muscles (*) or coelomocytes (**) were scored for Ncl phenotype to determine precisely the point of duplication loss in individual mosaics. All underlined cells were also scored for sexual phenotype by Nomarski microscopy as described⁶ (see legend to Fig. 2).

METHODS. To generate free duplications including the *her-1* region of LGV, 1115 wild-type F₁ progeny from γ -irradiated (4,000-6,500 rad) *him-8(e1489)IV*; *sma-1(e30) +/+ vab-8(e1017)V* hermaphrodites were singly plated and their F₂ progeny screened for absence of Sma or Vab phenotypes, indicating either: (1) a recombinant wild-type chromosome (for example, *sma-1 +/+ +*); (2) a lethal mutation tightly linked to one of the markers (for example, *sma-1 +/+ + vab-8 let*); or (3) presence of either a free or attached duplication complementing one of the markers in a homozygous mutant animal (for example, *sma-1 +/+ + sma-1 +/+ + Dp[sma-1(+)]*). These possibilities were distinguished by progeny testing. In one candidate that produced 60% wild-type and 40% Sma progeny, the presence of a free duplication, designated *ctDp8(V;f)*, was confirmed by cytological observation of small extrachromosomal fragments in DAPI-stained meiotic oocytes²⁰. *ctDp8* complemented the markers shown (excepting *vab-8(e1017)*, carried on the duplication); it also completely complemented the small *her-1* deficiency *y101hv1* (ref. 21) and caused no detectable sexual transformations in *her-1(+)* animals of either sex. To isolate an *sDp3-ctDp8* fusion duplication, 201 wild-type F₁ progeny from γ -irradiated (3,800 rads) *dpy-17(e164) III*; *sDp3(III;f)*; *him-8(e1489) IV*; *her-1(y101hv1) unc-42(e270) V*; *ctDp8(V;f)* hermaphrodites were singly plated and their F₂ progeny screened for the absence of both Dpy non-Unc and Unc non-Dpy individuals, suggesting physical linkage of *sDp3* and *ctDp8*. One such animal carried an apparent fusion duplication, designated *ctDp11*, which complemented *dpy-17, unc-36* and *ncl-1* as well as *her-1* and the other LGV markers shown (except *vab-8*). Its rate of mitotic loss per cell division was estimated to be $\sim 1/400$ (6 Ncl mosaics found among 200 L3 animals screened by Nomarski microscopy), which is similar to the rate observed for *sDp3* (ref. 22). *ctDp11* completely complemented the *her-1* null allele *y101hv1* and caused no detectable sexual transformations in



her-1(+) strains. *c*, Animals mosaic for *her-1* were identified as described below among self progeny of *dpy-17 ncl-1 unc-36 III*; *him-8 IV*; *her-1 unc-42 V*; *ctDp11* hermaphrodites or among Lon-2 (XO) cross progeny from males of the above genotype crossed to *dpy-17 ncl-1 unc-36 III*; *her-1 unc-42 V*; *lon-2 X* hermaphrodites. Among duplication-bearing cross progeny, 80% were males, indicating that *ctDp11* preferentially disjoins from the X chromosome in male meiosis. Probable mosaics were picked as phenotypic intersexes or as Unc non-Dpy or Dpy non-Unc animals and scored for Ncl phenotypes by Nomarski microscopy (the gene products of *unc-36* (ref. 22) and *unc-42* (this work) are required in some cells derived from the ABp lineage, and the *dpy-17* gene product is required in certain cells derived from both the AB and P₁ lineages^{22,23}). In addition, wild-type L3 larvae were screened directly for Ncl mosaicism by Nomarski microscopy. Many of the Unc non-Dpy and Dpy non-Unc animals carried meiotically rearranged or recombinant duplications, which were easily distinguishable from the desired mosaics because all scoreable cells were either Ncl or non-Ncl in any given animal. Although mitotic recombination or loss of part of the fusion duplication theoretically could produce Ncl mosaic animals that would be *her-1(+)* or *her-1(-)* in all cells, sensitive screens have failed to detect mitotic recombination in *C. elegans* ($<10^{-5}$ per cell division; C.P.H., unpublished results), and no evidence was seen for mitotic breakdown of *ctDp11*. Additionally, intersexual phenotypes similar to those in Table 1 were observed in *her-1* mosaics using the duplication *ctDp8* and the *osm-6* phenotype as a cell-autonomous marker²⁴.

TABLE 1 Sexual phenotypes of post-embryonic blast cells in XO *her-1 ncl-1* mosaics

Point of Dp loss	No. of animals	Masculinized <i>her-1(-)</i> cells	Feminized <i>her-1(+)</i> cells	Point of Dp loss	No. of animals	Masculinized <i>her-1(-)</i> cells	Feminized <i>her-1(+)</i> cells
(1) AB	2*	V5L V5R V6L V6R TL TR B P10		(26) P ₁	1§	no vulva [M]	V5L V5R V6L V6R TL TR B [P10]
(2) AB	1*	V5L V5R [V6L] [V6R] B [P10]		(27) P ₂	5	Z2 Z3	
(3) ABa	1†‡	V6L [V6R]	[TL] [TR]	(28) P ₂	1	Z2 Z3	[M]
(4) ABa	1†‡	V6L V6R	[TL]	(29) P ₂	1	Z2 Z3	[disorganized rays]
(5) ABa	1†	V6L V6R		(30) P ₃ /D	1	(Z2 Z3)	
(6) ABp	2*	V5L V5R TL TR B P10		(31) P ₃ /D	1§¶		B[M] E (Z2 Z3)
(7) ABp	1†‡	V5L V5R TL TR B P10		(32) P ₃ /D	1†	(Z2 Z3)	[M]
(8) ABpl	2*	V5L TL (P10)		(33) C	1		
(9) ABpl	1*‡	V5L TL (P10)		(34) EMS/MS	1§		V5L V5R V6L V6R TL TR [B] (E)
(10) ABpl	1*	V5L (P10)	[V6R]	(35) EMS/MS	1§	[Z1] [Z4]	Z2 Z3
(11) ABpla	1†	V5L TL (P10)					V5L V6L [V6R] TL P10 (E)
(12) ABplap	1†	V5L TL (P10)		(36) ?	1§#		V5L V5R V6L V6R TL TR [B] M Z1
(13) ABplap	1†‡	V5L TL (P10)					Z4 (Z2 Z3)
(14) ABplapap	1*‡	V5L P10		(37) ?	1§#		V5L V5R V6L [V6R] TL [B]
(15) ABpr	1*	V5R TR B (P10)					[P10] M Z1 Z4 (Z2 Z3)
(16) ABpr	1†	V5R TR B (P10)		(38) ?	1§#		V5L V5R V6L V6R [TR] B [P10] M
(17) ABpr	2*‡	V5R TR B P10					Z1 Z4 (Z2 Z3)
(18) ABpr	1*	V5R TR B (P10)	V5L [V6L] TL				V5L V5R V6L V6R [TR] B [P10] M
(19) ABpr	1†	[TR] B (P10)	V6R	(39) ?	1§#		Z1 Z4 (Z2 Z3)
(20) ABpra	1†‡	V5R TR (P10)					V5L V5R V6L [V6R] TL TR B
(21) ABprapa	1†	V5R (P10)		(40) ?	1†§#		[Z1 Z4] (Z2 Z3)
(22) ABprapa	1†	V5R P10					V5L V5R V6L V6R [B] [Z1] [Z4]
(23) ABprapap	2†	V5R P10					(Z2 Z3)
(24) P ₁	1§		V5L V5R [V6L] [V6R] B P10	(41) ?	1†§#		(Z2 Z3)
(25) P ₁	1§		V5L V5R V6L V6R TL [TR] B [P10]				

Mosaic animals were identified either by morphological phenotype or *ncl-1* mosaicism (see footnotes), and sexual phenotypes were scored by Nomarski microscopy⁶. The point of duplication loss was determined by scoring a variety of cells for the Ncl phenotype (see text and Fig. 1c). Cell genotypes were assigned directly for scoreable cells or inferred from the genotypes of their relatives, assuming the minimum number of duplication losses consistent with the observed Ncl phenotypes in each animal (see below). Cells listed are only those sexually dimorphic blast cells that showed sexual fates inconsistent with their *ncl-1 her-1* genotype; that is, *her-1(-)* cells that followed hermaphrodite fates and *her-1(+)* cells that followed male fates are not shown. But dimorphic cells were scored in all animals listed, so that the complete sexual phenotype of each animal can be ascertained from the data by reference to Fig. 1c. The mosaics in lines 1, 5–9, 11–17, 20–23, 27, 29, 30 and 33 developed as complete males, that is, all *her-1(-)* as well as *her-1(+)* cells followed male fates. Because the Ncl phenotype cannot be scored in the intestine (E lineage) or the germline (Z2, Z3), we could not distinguish EMS from MS losses or P₃ from D losses. Neither could we be certain that the apparent P₂-loss mosaics (lines 27–29) were not produced by consecutive losses in C and D, resulting in a *her-1(+)* rather than *her-1(-)* germ line (although we have no evidence for consecutive losses of *ctDp11* elsewhere in the lineage). Because of these ambiguities, the genotype (Ncl phenotype) was not or could not be scored directly or inferred from scored relatives for some cells, which are shown in parentheses in the columns consistent with their sexual phenotypes (masculinized or feminized). Cells shown in square brackets followed intersexual fates, that is, gave rise to structures that showed both male and hermaphrodite features. Seven scored mosaics died as larvae (including duplication losses at AB, ABa, ABp, ABal, ABpl, EMS/MS), probably as the result of severe defects in defecation associated with intersexual phenotypes.

* Mosaic animals initially identified by Unc non-Dpy phenotype (presumed Dp loss in ABp lineage).

† Mosaic animals initially identified as *ncl-1* mosaics by Nomarski microscopy.

‡ Lon-2 cross-progeny indicating that the animal is XO (see legend to Fig. 1c).

§ Mosaic animals initially identified as either abnormal males or intersexual hermaphrodites.

|| Mosaic animals initially identified by Dpy non-Unc phenotype (presumed Dp retention in ABp lineage, loss in a P₁ lineage).

¶ This phenotype could be explained by a second Dp loss in the E lineage.

These animals made yolk protein (except for 39) and therefore are suspected to have lost the duplication in the E lineage; see text. Determination of germ line genotype by progeny observation was not feasible because these animals were nearly or completely sterile. Therefore, duplication loss in P₄ was not ruled out, but this could not explain the phenotype, as loss at P₂ (lines 27–29) did not cause extensive feminization of *her-1(+)* cells.

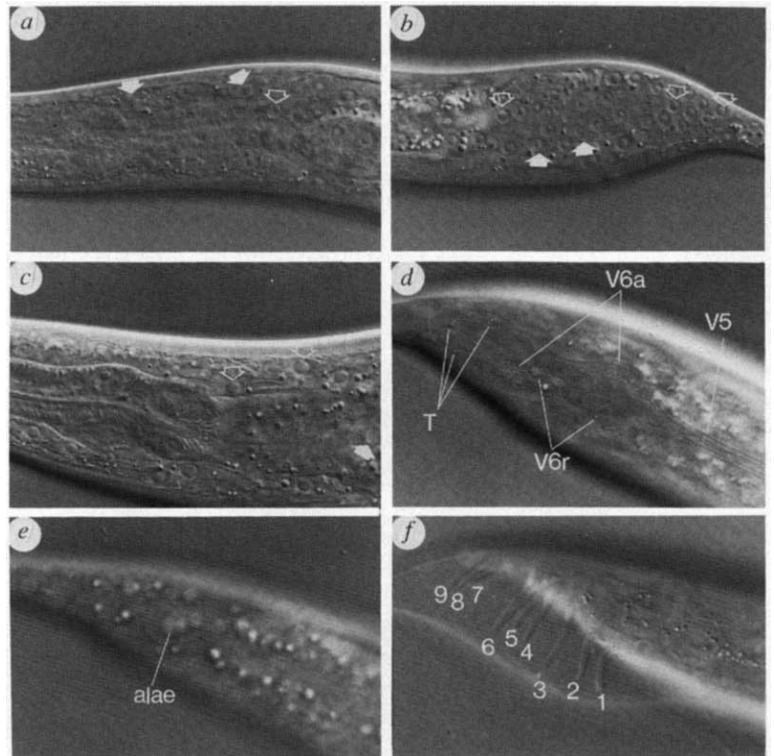
predominantly feminization of the remaining *her-1(+)* cells by the resulting *her-1(-)* cells. Although it is difficult to rule out the possibility that some cases of masculinization resulted from perdurance of *her-1* gene product following a duplication loss, rather than from non-autonomy, the observed feminization is inconsistent with this explanation.

Our results indicate that *her-1* function in sexually dimorphic cells is neither necessary nor sufficient for their male development. We conclude that *her-1* or a *her-1*-regulated activity can function non-cell-autonomously, which means that cell interactions can play a part in determining sexual phenotypes. We can also conclude that *her-1* is expressed in many cells, because duplication losses in ABa, ABpr, ABpl, EMS or MS, P₃, and probably E result in intersexual phenotypes.

A model for *her-1* action must explain both the masculinization of *her-1(-)* cells and the more puzzling feminization of *her-1(+)* cells in mosaics, as well as the variability of mosaic phenotypes. Previous genetic analysis¹ strongly suggests that the *her-1* gene product dictates male development by negatively regulating the function of the *tra-2* gene (Fig. 1a). Therefore the cell interactions we observe are most probably occurring in the regulation of *tra-2* function by *her-1* function. One simple model that would explain our results is presented in Fig. 3.

It is difficult to assess whether the observed non-autonomy can result from systemic effects or is restricted to short-range interactions only, because the spatial relationships of cells are continually changing during embryogenesis, and we do not know when determinative interactions between *her-1(-)* and *her-1(+)*

FIG. 2 Nomarski photomicrographs of *ncl-1 her-1* mosaics. Derivation of the sexually dimorphic structures scored in such animals is as follows¹¹ (refer to Fig. 1c): V5, V6 and T produce the tail sensory rays 1, 2–6, and 7–9, respectively (right and left), in males (f) and posterior extensions of the lateral alae in hermaphrodites (e). B produces the spicules in males and does not divide in hermaphrodites. The posterior daughter of P10 (P10p) produces the hook in males and does not divide in hermaphrodites. M produces tail sex muscles in males and mid-ventral vulval sex muscles in hermaphrodites. Z1 and Z4 give rise to the single-lobed somatic gonad in males and the bilobed gonad in hermaphrodites. Z2 and Z3, the primordial germ line cells, give rise to sperm only in males and to both sperm and oocytes in hermaphrodites. For the examples shown, anterior is to the left in a, b and c, and to the right in d, e and f. a, Post-pharyngeal region of an XO *Lon* mosaic L3 larva that developed into a complete male (Table 1, line 6), showing non-Ncl (*her-1*(+)) MS-derived muscles (solid arrows) and Ncl (*her-1*(–)) ABp-derived neurons (open arrow). (Corresponding cells in a and c must be compared, because nucleolar sizes vary with cell type in either a Ncl or non-Ncl background.) The pattern of Ncl and non-Ncl nucleoli in this panel and in b (as well as in other cells not shown) indicate loss of the duplication in the ABp cell. b, Tail of the same animal as in a, showing non-Ncl and Ncl ray-cell groups. Selected non-Ncl ABa-derived V6 ray precursor cells and Ncl ABp-derived V5 and T ray precursor cells are indicated by solid and open arrows, respectively. c, Post-pharyngeal region of an adult mosaic intersexual animal (Table 1, line 24) showing Ncl MS- and D-derived muscles (open arrows) and a non-Ncl AB-derived neuron (solid arrow) (compare with a). The pattern of Ncl and non-Ncl nucleoli in these and other cells indicates loss of the duplication in P₁. d, Tail of the same animal as in c, showing intersexual development of *ncl-1*(+) (therefore *her-1*(+)) tail structures. V5R-derived cells followed a completely hermaphrodite fate (alae). V6R-derived cells followed both hermaphrodite (alae, V6a) and male (2 rudimentary rays, V6r) fates. TR-derived cells followed



male fates (3 rudimentary rays) (compare with e and f). e, Wild-type adult hermaphrodite tail, showing posterior extensions of lateral alae. f, Wild-type adult male tail showing right side rays 1–9.

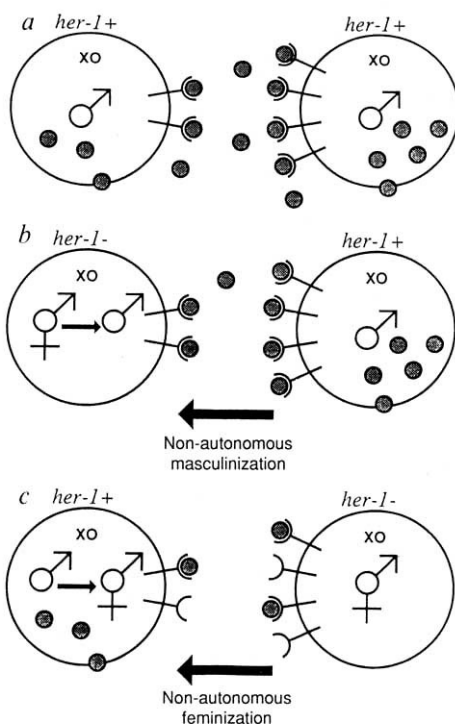


FIG. 3 A model for non-autonomous *her-1* action through paracrine effects of a secreted ligand. a, In wild-type XO animals, the ligand, possibly the *her-1* product (shaded circles) is postulated to bind to and inhibit the function of a cell-surface receptor, expressed independently of *her-1* activity and possibly encoded by *tra-2* (see text). Such a ligand could act in an autocrine or paracrine fashion. Consistent with these roles for *her-1* and *tra-2* are

findings that alleles of both these genes result in dose-dependent phenotypes²⁵, that *her-1* is transcribed only in XO animals²¹ and that *tra-2* is expressed in both XX and XO animals²⁶, as well as the recent molecular characterization of the *her-1* (M. D. Perry *et al.*, manuscript in preparation) and *tra-2* (P. Kuwabara and J. Kimble, personal communication) gene products. b, Non-autonomous masculinization in XO *her-1* genetic mosaics can be explained by *her-1*(+) cells producing enough ligand to masculinize both themselves and neighbouring *her-1*(–) cells. c, Non-autonomous feminization in XO *her-1* genetic mosaics can be explained by titration of ligand by unoccupied receptors on neighbouring cells. The proposed inactivation of receptor function by binding of its natural ligand would be unusual, but not without precedent. For example, the inhibitor of the insulin receptor tyrosine kinase, *pp63* (ref. 27), and the interleukin-1 receptor antagonist²⁸ are both naturally occurring ligands that bind to and inhibit their cognate receptors. The Mullerian inhibiting substance (MIS) appears to act in mammalian male gonadal development by binding to and inhibiting an epidermal growth factor-tyrosine kinase receptor²⁹. The *Drosophila* pattern formation gene *patched* (*ptc*) has been proposed to encode a constitutively active integral membrane protein that is inactivated by the binding of a putative ligand encoded by *hedgehog* (*hh*)³⁰. Genetic mosaic analysis of *ptc* and *hh* show examples of non-autonomy similar to that observed for *her-1*, in which clones of mutant cells induce the mutant phenotype in neighbouring wild-type cells^{31,32}. Our observation that duplication losses in the AB lineage show predominantly masculinization of *her-1*(–) AB-derived cells, whereas P₁-loss mosaics show predominantly feminization of *her-1*(+) AB-derived cells can be interpreted to mean that the P₁-derived cells of the gonad and intestine in general produce more of the ligand and the receptors than do AB-derived cells. Therefore, duplication loss in P₁ lineages leads to a large excess of empty receptors, whereas AB loss results in a relatively small number of empty receptors that can be filled by ligand produced in P₁-derived cells. An excess of empty receptors in both types of mosaics would explain the observed variability of mosaic phenotypes: as the amount of secreted ligand available for masculinization of *her-1*(–) cells would be limiting, it could be distributed among the receptors of different cells in different animals. In AB-loss mosaics, the larger amount of ligand available for redistribution relative to the small excess of empty receptors could explain why early losses in the AB lineage generally result in no greater number of feminized cells than do later losses.

cells are taking place. Earlier temperature-shift experiments¹² and preliminary molecular data (ref. 13; M. D. Perry *et al.*, manuscript in preparation) indicate that *her-1* expression begins during the first half of embryogenesis, when no cells are separated by more than a few cell-diameters. But *her-1* continues to be expressed and required for maintenance of male sexual differentiation of the intestine and germ line into adulthood¹⁴. Secretion of *her-1*-encoded ligand into the pseudocoelom by the intestine, for example, could exert masculinizing effects on post-embryonic lineages in many parts of the animal. The finding that some postembryonic blast cells in mosaic animals give rise to intersexual lineages is consistent with the view that *her-1* can still influence sexual fates post-embryonically after the onset of blast-cell divisions, or that intermediate levels of *her-1* can result in partial masculinization.

Our results provide one more demonstration (others reviewed in ref. 15) that the sex-determining mechanisms in *C. elegans* and *Drosophila* differ fundamentally. The suggested model is more reminiscent of primary sex determination in mammals, where gonadal supporting cells in a chimaeric male that fail to express the testis-determining gene can be masculinized by ligand (MIS) from nearby Sertoli cells^{16,17}. Likewise, the non-autonomy of *her-1* action in *C. elegans* could provide an error-correcting mechanism, whereby a cell in an XO animal that aberrantly assessed its X/A ratio and failed to express *her-1* would be masculinized by ligand from surrounding cells, thereby ensuring development of a complete set of male-specific structures required for mating. □

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Yeast *RAD14* and human xeroderma pigmentosum group A DNA-repair genes encode homologous proteins

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XERODERMA pigmentosum (XP), a human autosomal recessive disorder, is characterized by extreme sensitivity to sunlight and high incidence of skin cancers. XP cells are defective in the incision step of excision repair of DNA damaged by ultraviolet light. Cell fusion studies have defined seven XP complementation groups, XP-A to XP-G (refs 1, 2). Similar genetic complexity of excision repair is observed in the yeast *Saccharomyces cerevisiae*. Mutations in any one of five yeast genes, *RAD1*, *RAD2*, *RAD3*, *RAD4*, and *RAD10*, cause a total defect in incision and an extreme sensitivity to ultraviolet light³. Here we report the characterization of the yeast *RAD14* gene. The available *rad14* point mutant is only moderately ultraviolet-sensitive, and it performs a substantial amount of incision of damaged DNA^{4,5}. Our studies with the *rad14* deletion (Δ) mutation indicate an absolute requirement of *RAD14* in incision. *RAD14* encodes a highly hydrophilic protein of 247 amino acids containing zinc-finger motifs, and it is similar to the protein encoded by the human XPAC gene that complements XP group A cell lines⁶.

The *RAD14* gene in the 9-kilobase (kb) yeast DNA insert in plasmid pBM127 was located by examining the complementation of the *rad14-2* mutation by different DNA subclones (Fig. 1). The 1.4-kb *HindIII*-*XbaI* fragment in plasmid pBM149 (Fig. 1a) partially complemented the *rad14* mutation (Fig. 1b),

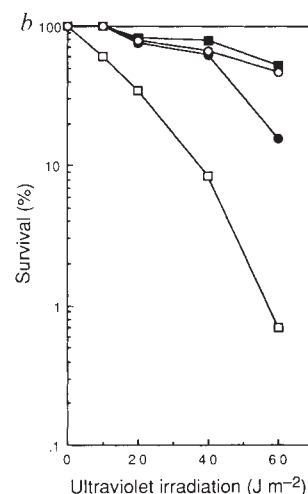
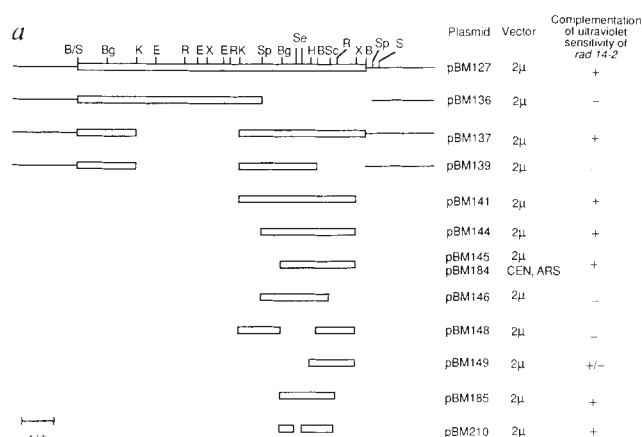
whereas plasmid pBM210, in which the 205-base-pair (bp) *SpeI* fragment of pBM185 had been deleted, fully complemented the *rad14* mutation, indicating that the *RAD14* complementing activity is located in the 1.05-kb *SpeI*-*EcoRV* DNA fragment. The sequence of the *rad14* complementing *SpeI*-*EcoRV* DNA fragment contains a single long open reading frame (ORF) which starts with the ATG codon at position +1 and extends to the TAA termination codon at position +742 (Fig. 2). The *RAD14* ORF encodes a highly hydrophilic protein of 247 amino acids with a predicted relative molecular mass of 29,328. It contains 22.3% basic, 20.6% acidic, 26.7% polar, and 30.4% nonpolar residues and has a pI of 6.3. A zinc-finger motif CX₂CX₁₈CX₂C is present in *RAD14* from amino-acid residues 67 to 92 and another potential metal-binding sequence HX₃HX₂₃CX₂C occurs at residues 207–238.

RAD14 has homology to the human XPAC protein which contains 273 amino acids⁶ (Fig. 3). XPAC resembles *RAD14* in being very hydrophilic and in possessing a high proportion of glutamic acid, 13.4% in *RAD14* and 12.4% in XPAC, and lysine, 11.7% in *RAD14* and 10.6% in XPAC. The homology is most extensive in the middle portion of the two proteins, encompassing residues 67 to 198 in *RAD14* and 105 to 235 in XPAC. In this region the two proteins share 33% identical residues, and 61% of the residues are similar if identical and conserved residues are grouped together. Overall, the two proteins share 27% identical and 54% similar residues. The homology between the two proteins is highly significant, as indicated by the adjusted score of 14 (ref. 7). The 4-cysteine (4C) zinc-finger motif is present in both proteins at the beginning of this homologous segment. The zinc-finger sequence in *RAD14* and XPAC might be involved in DNA binding or it might affect protein-protein interactions⁸. Towards the carboxyl terminus, *RAD14* contains the sequence HX₂HX₂₀CX₂C between residues 211 to 238, and human XPAC contains a sequence HX₂HX₁₆CX₂C between residues 242 to 264 which could also be involved in metal binding. But because one patient homozygous for a nonsense mutation of the XPAC Arg 228 codon has only mild XP symptoms (K. Tanaka, personal communication), the carboxyl terminus of XPAC and, by implication, of *RAD14*, may not be essential for function. The amino-terminal region, making up

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FIG. 1 a, Restriction map of the 9-kb yeast DNA insert in plasmid pBM127 and complementation ability of DNA fragments cloned into yeast vectors YEplac195 (2 μ) and YCplac33 (CEN, ARS)¹⁵. Open bars, yeast DNA containing *RAD14* and flanking sequences; thin lines, YEp24 sequences; + and -, complementation of the ultraviolet sensitivity of the *rad14-2* mutation in yeast strain LP2899-8A (*MAT α rad14-2 ura3-52 trp1-289 ade2-1*). B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; R, *Eco*RV; S, *Sal*I; Sc, *Sac*I; Se, *Spe*I; Sp, *Sph*I; X, *Xba*I; B/S, *Bam*HI-*Sau*3A cloning site of YEp24. Deletion of the *Sph*I and *Kpn*I fragments of pBM127, generated plasmids pBM136 and pBM137, respectively. Deletion of the *Bam*HI fragment of pBM137 yielded plasmid pBM139. Only plasmid pBM137 retained *rad14*-complementing ability. The 3.5-kb *Kpn*I-*Xba*I, 2.7-kb *Sph*I-*Xba*I, 2.25-kb *Bgl*II-*Xba*I and 1.65-kb *Bgl*II-*Eco*RV fragments were cloned into YEplac195 yielding plasmids pBM141, pBM144, pBM145 and pBM185, respectively. All these plasmids complemented the ultraviolet sensitivity of the *rad14* mutation. b, Survival after ultraviolet irradiation in *rad14-2* strain LP2899-8A with various plasmids. $\square$, LP2899-8A (*rad14-2*) + vector YEplac195; $\blacksquare$, LP2899-8A (*rad14-2*) + pBM127; $\circ$, LP2899-8A (*rad14-2*) + pBM184; $\bullet$, LP2899-8A (*rad14-2*) + pBM149. Strains were grown on synthetic complete medium lacking uracil (SC-ura) for maintaining selection for plasmids. Plasmids pBM127 and pBM184 restore wild-type levels of ultraviolet resistance to the *rad14-2* strain.

METHODS. The moderate ultraviolet sensitivity of the *rad14-2* mutant made screening for ultraviolet-resistant clones difficult after transformation with a yeast gene pool. We overcame this problem by taking advantage of the synergistically increased ultraviolet sensitivity of the *rad14-2 rev2-1* double



mutant. The strain BM1281-12D (*MAT α rad14-2 rev2-1 ura3-52 arg4-17 ade2-1 lys2-1 trp2*) was transformed with the yeast recombinant plasmid library in YEp24 (ref. 16) to Ura⁺. About 4,000 transformants were replica-plated onto SC-ura medium and irradiated with 60 J m⁻² ultraviolet light. Seven ultraviolet-resistant clones were isolated and corresponding clones from the unirradiated master plate were transferred onto synthetic complete medium which had been supplemented with 5-fluoro-orotic acid to promote plasmid-loss¹⁷. In all cases, a Ura⁻ phenotype was concomitant with the loss of ultraviolet resistance, indicating that ultraviolet resistance was plasmid-borne. Plasmid DNA from ultraviolet-resistant yeast strains was isolated and transformed into *rad14* and *rev2* mutant strains to discriminate between plasmids containing the *RAD14* or the *REV2* gene. Five of these plasmids complemented the *rad14* mutation and the two remaining plasmids complemented the *rev2* mutation. All *rad14*-complementing plasmids contained the same 9-kb yeast DNA insert. Ultraviolet survival was determined as described previously¹⁸.

Yeast RAD14	1	MQNLNG..GYINPKDKLPNSDFTDDQFESEFGSKKQKTLQDWKKEQLER	48
Human XPAC	1	MAAADG..ALPEAAALEQPAELPA..SVRASIERKRQALM.LRQARLAA	45
Mouse XPAC	1	--T-EEKQTS--PV-ADE--Q---.A---V-----	47
Yeast RAD14	49	KMLYENAPPP....EHISKAPK.....	66
Human XPAC	46	RPYSATAAAATGGMANVKAAPKIIDTGGGFIEEEEEEEQKIGKVHQPQ	95
Mouse XPAC	48	---P..-----V-S-----M---K-----KHE.--NI--E--	94
Yeast RAD14	67	CIECHINIEMDPVLHDFKLQVCKQCSKEHPEKYALLTKTE	107
Human XPAC	96	PVMEFDYVICEECGKEF.MDSYLMNHFDLPTCDNC.RDADDKHKLITKTE	143
Mouse XPAC	95	-----T-----S-----	142
Yeast RAD14	108	CKEDYFLTDPELND.EDLFHRLEKPNPHSGTFARMQLFVRCEVEAFAFKK	156
Human XPAC	144	AKQEYLLKDCDLEKREPPLKFIVKKNPHHSQWGMKLYLKLQIVKRSLEV	193
Mouse XPAC	143	-----A-R-L-----R-----V---A---	192
Yeast RAD14	157	WGGEGLDEEWQRREEGKAHRREKKYKIKEMRLKTRAQEYTNRLREKK	206
Human XPAC	194	WGSQEALEEAKEVRQENREKMKQKFKDKVKELRRRAVRSSVW.....KR	237
Mouse XPAC	193	-----D-----I-----	236
Yeast RAD14	207	HGKAHIHHSFSDPVDGGIDEDGYQIQRRCCTDCGLETEEIDI	247
Human XPAC	238	ETIVHQHEYG..PEENLEDDMY...RKTCTMCGHELTYEKM	273
Mouse XPAC	237	--TT-----L-----	272

FIG. 3 Protein sequence alignment between yeast *RAD14* and human and mouse *XPAC* gene products. The Bestfit program of the GCG Sequence Analysis Software Package (Genetics Computer Group, Madison) was used for alignment. Sequence similarity is shown between yeast *RAD14* and human *XPAC* proteins, where a vertical line represents amino-acid identity and a colon represents amino-acid similarity as defined by the computer program using the default parameters. Gaps introduced into the sequence for optimal alignment are signified by a dot. The dashes in the mouse *XPAC* sequence represent identity to the human *XPAC* sequence at that position. The numbering indicates the amino-acid residue at the beginning and end of each line, respectively. The cysteine and histidine residues in the potential metal-binding motifs present in both *RAD14* and human *XPAC* are marked by an asterisk.


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-708 GATCATAATGGGATACTTCGTTGATAATAAGCAGAATCAACATAGCAGACTGCTAGTGGCATACTCCACAACCCCTGTTTTTTTTTTTTT
-618 TTTTGTGTTGAATAACTGTACTTCTATAGAAGCTCTATCTACAGCATGTTTGACGTTTGCTAAGTTGTAGGGAGAAAAGAGGAAAGTGA
-528 AGAAGGTAAGGAATTTATCGGAAGTAAAGAGTTTGGATCTTCGTAGTGAAGGTATCGAACGTAACGCTATGACTCCCGAACAAAAG
      SpeI
-438 GCCAAAGCTAGTATGTGTGAAATGATTCTGTGTTTGTATTTTAAACCGTGGGTTTCTTTTACTAACAAATTTAACGATGAGATGAGCTGTG
-348 CAGGAGGCTAACAGGAAATTAGCAATAGAACGGTTAAGAAAAAGGGAACTACTGAGTAGCGACCAATTGAATCGAATAGAAAGTAGGAAT
      SpeI
-258 GAACCTTTAAAAACCCGGCCTCTCGCAGTTACTAGTGGCAGCAATCGGGATGATAATGCAGCAGCCGAGTACATGTGCCAAATCATAAT
-168 GGACAACCGTCTGCGCTTGCTAACACTAACACTACTTCACTTTATGGTAGCGGAGTAGTTGATGGAAGTAAAGGGATGCGTCG
-78 GTACTCGACAAAAGGCCAACGGATAGAATCAGACCTAGCATAAGGAAACAAGATTACATTGAGTACGATTTTGCCACCATGCAGAACTTG
                                     M Q N L 4
      HindIII
13 AATGGTGGTTATATCAACCTAAGGACAAGCTTCCAAATCTGACTTTACCGATGACCAAGAATTTGAATCTGAGTTGGATCTAAAAAG
   N G G Y I N P K D K L P N S D F T D D Q E F E S E F G S K K 34
103 CAGAAGACACTACAGGACTGGAAAAAGGAACAACCTGAACGGAAAAATGCTGTACGAAAAATGCACCTCCTCCAGAGCATATTTCAAAGGCG
   Q K T L Q D W K K E Q L E R K M L Y E N A P P P E H I S K A 64
      BamHI
193 CCGAAATGTATTGAATGTCATATTAATATTGAGATGGATCCTGTGCTACATGATGTGTTCAAGTTACAAGTTGTAAACAGTGTCTAAG
   P K C I E C H I N I E M D P V L H D V F K L Q V C K Q C S K 94
283 GAGCATCCAGAAAAGTATGCACTACTGACGAAAAAGAAATGTAAGGAAGATTACTTTTAAACAGACCCCGAATTGAATGATGAGGATCTC
   E H P E K Y A L L T K T E C K E D Y F L T D P E L N D E D L 124
373 TTTCATAGACTAGAAAAGCCGACCCCTCATTCGGGGACATTTGCAAGAATGCAACTATTGTTAGATGTGAAGTGGAGCCTTTGC GTTC
   F H R L E K P N P H S G T F A R M Q L F V R C E V E A F A F 154
463 AAGAAATGGGTGGAGAAGAAGGTTTAGATGAGGAATGGCAACGTCGTGAAGAAGGAAAGGCTCACAGAAGGGAGAAAAATACGAAAAG
   K K W G G E E G L D E E W Q R R E E G K A H R R E K K Y E K 184
      SacI
553 AAAATCAAGGAAATGCGACTGAAAAAGAGCTCAAGAATATACTAATAGATTAAGAGAAAAGAAGCATGGGAAAGCCCATATTCATCAT
   K I K E M R L K T R A Q E Y T N R L R E K K H G K A H I H H 214
643 TTTAGTGATCCAGTTGATGGAGGTATTGATGAAGACGGTTATCAAATCAAAGAAGAAGATGTACAGACTGCGGGCTAGAACTGAAGAA
   F S D P V D G G I D E D G Y Q I Q R R R C T D C G L E T E E 244
      EcoRV
733 ATTGACATTTAAATGTTGGTTATGTATATAAGAATATAACAAGAAAGTCATAATAAGGTGTTTGTATTGTGTTTCATTGATTGATATCCCA
   I D I *
823 TTTGTCACCTCGTACATAAATTAACCTATCTCGGAAAACTGGTTTGAAATCTTTTATTCATCCACCTATTTAATAATATGATTACAATCA
913 ATTCCTTGGGTGCATAAAATTACACCTATTCTTGCTTCTCTTCTCGCCTGATAAAGTTGTTTTGCCTTATATGTTTATAGTTTTTAATA
1003 TTTATTTGAAGGCCACATGGGGCCTCTTTCCCCCATTTGGTTGAATAAGTATCCCTGTAGTGGCCTAAAATGCTAAAAATAAATGAAC
1093 CTACACCTAGCATACCCGAGAAATATACCAATGGCATTGGAGGTTAAAAATGAGGTATTTTGTCTTTTAAATGGCATTCTGCTTGTGTC
1183 TGTTTTCCAGGCTCCTCCTCACTAACTTCTCAGTGATCTTTTCTTATTAGAGGATGGGTCGTCAGTGCTGGTCGCTGACGATGTTGGGT
1273 TCAAAGTTTCTGTTGGAAGCATCATTGGTGGTCTATAAGCCAAATATAGTGGTTGCATTGGAGTACCATCATATTGGTACAATTGCAGGG
      NcoI XbaI
1363 TGTAATAACCATGGTAAGGATCTATACCTACGGCATATCTCTTGAAAGTTTCTGTTTGGTTATATCTAGA

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FIG. 2 Nucleotide sequence of the 2,140-bp *Sau3A*–*XbaI* DNA fragment containing the *RAD14* gene. Numbers on the left correspond to the DNA sequence and numbers on the right correspond to the protein sequence. The cysteine and histidine residues that might be involved in metal binding are circled.

METHODS. DNA fragments, obtained by digestion with various restriction

enzymes were cloned into M13 derivative phages and pUC derivative plasmids. The nucleotide sequence of both strands of the *RAD14* gene was determined by the chain-termination method¹⁹ using 5'-(α -[³⁵S]thio) triphosphate²⁰. DNA fragments in pUC-plasmids were sequenced using denatured double-stranded DNA templates²¹.

residues 1–66 in *RAD14* and residues 1–104 in human *XPAC*, has diverged considerably. In this region, the human and mouse *XPAC* proteins also differ much more than in the remainder of the sequence. Furthermore, human *XPAC* complementary DNA clones that encode a truncated protein missing the 58 N-terminal residues restore the DNA repair defect of *XP-A* mutant cell lines close to wild-type levels⁶. These observations indicate that the amino terminus of *XPAC* is not absolutely required for function. Our observation that plasmid pBM149 complements the ultraviolet sensitivity of *rad14* to a high degree (Fig. 1b) suggests that the amino terminus of *RAD14* is also not essential for function. Plasmid pBM149 is missing the *RAD14* coding sequence for the first 13 amino acids, but because the

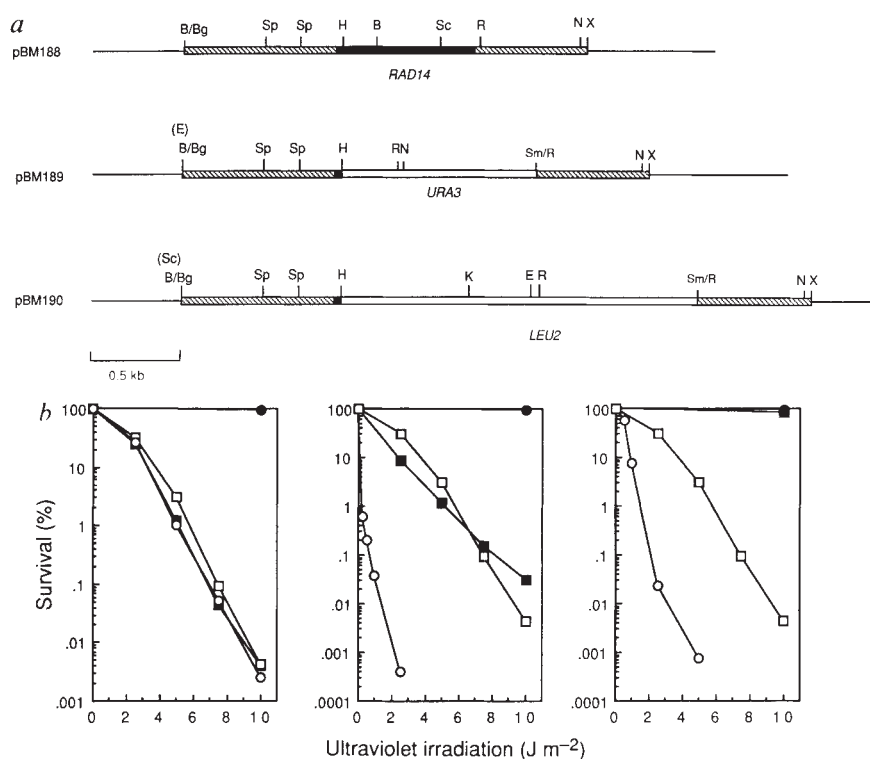
next ATG codon where translation might initiate occurs at nucleotide +148, this protein may actually be deleted for the first 49 amino acids.

Genetic studies with deletion mutations have revealed additional roles for some of the yeast excision repair genes in viability^{9,10} and mitotic recombination^{11,12}. To define the biological roles of *RAD14* unambiguously, we constructed a genomic deletion mutation of the *RAD14* gene by replacing *RAD14* sequences from positions +40 to +814 with either the yeast *URA3* or *LEU2* gene (Fig. 4a). We found no effect of the *rad14Δ* mutation on growth or mitotic intrachromosomal recombination of a *his3 Δ3' his3 Δ5'* duplication¹¹.

FIG. 4 *a*, Plasmids for creating a genomic deletion mutation of the *RAD14* gene. Restriction map of the 2.25-kb DNA fragment encompassing the *RAD14* gene in pBM188 and replacement of the *RAD14* gene by *URA3* in pBM189 and by *LEU2* in pBM190. The *EcoRI* (E) and *SacI* (Sc) sites in the polylinker region in pBM189 and pBM190 are in parentheses. Symbols for restriction sites as in Fig. 1a with the following additions: N, *NcoI*; Sm, *SmaI*. Solid bars, *RAD14* ORF; hatched bars, 5' and 3' flanking regions of the *RAD14* gene. The *URA3* and *LEU2* genes are shown by open bars, and thin lines represent pUC19 sequences.

b, Survival after ultraviolet irradiation of *RAD*⁺ strain DBY747 (*MATa ura3-52 leu2-3,-112 trp1-289 his3-Δ1*) and its isogenic derivatives bearing deletions of various *RAD* genes. Left, ●, *RAD*⁺; □, *rad14Δ*; ■, *rad1Δ*; ○, *rad14Δ rad1Δ*. Middle, ●, *RAD*⁺; □, *rad14Δ*; ■, *rad6Δ*; ○, *rad14Δ rad6Δ*. Right, ●, *RAD*⁺; □, *rad14Δ*; ■, *rad52Δ*; ○, *rad14Δ rad52Δ*. METHODS. Plasmids for creating a genomic *rad14Δ* mutation were constructed as follows. A 2.25-kb *BglII-XbaI* fragment from positions -844 to +1,427 containing the entire *RAD14* ORF and 5' and 3' flanking regions was cloned into *BamHI-XbaI* digested pUC19, where the *HindIII* site of the polylinker had been removed, generating plasmid pBM188. The *HindIII-EcoRV* fragment of pBM188 from positions +40 to +814 (Fig. 2), which contains most of the *RAD14* ORF, was then replaced by either a 1.1-kb *HindIII-SmaI*-fragment containing the yeast *URA3* gene, or a 2.0-kb *HindIII-SmaI*-fragment containing the yeast *LEU2* gene, generating plasmids pBM189 and pBM190, respectively, which delete all but the first 40 nucleotides of the *RAD14* ORF. Transformation of *RAD*⁺ strains to Ura⁺ with the 2.6-kb *EcoRI-XbaI* DNA fragment of pBM189 resulted in a *rad14Δ::URA3* strain, whereas transformation of *RAD*⁺ strains to Leu⁺ with the 3.4-kb *SacI-NcoI* DNA fragment of pBM190 generated a genomic *rad14Δ::LEU2* strain. Allelism between the *rad14Δ* and *rad14-2* mutation was established by testing the ultraviolet sensitivity of *rad14Δ/rad14-2* diploids, and sporulation of these diploids produced only ultraviolet-sensitive spores. Southern analyses were carried out by hybridizing either the 541-bp *RAD14* internal *HindIII-SacI* fragment or the 613-bp flanking *EcoRV-XbaI* fragment to genomic DNA isolated from *RAD*⁺ and isogenic *rad14Δ* strains.

The *rad14Δ* mutation generates very high ultraviolet sensitivity equivalent to that of the totally incision-defective *rad1Δ* mutant (Fig. 4b). Our studies with the much less ultraviolet-sensitive *rad14-2* mutant indicated an epistatic relationship between *rad14* and mutant genes representing each of the three different DNA repair epistasis groups¹³. This ambiguous result is probably due to leakiness of the *rad14-2* point mutation, because studies with the *rad14Δ* mutation clearly indicate an epistatic relationship between *rad14Δ* and *rad1Δ* mutant genes, whereas a synergistic decline in ultraviolet survival was observed when the *rad14Δ* mutation was combined with the *rad6Δ* mutation defective in postreplication repair¹⁴ (Fig. 4b). Even though the *rad52Δ* mutation does not cause any increase in ultraviolet sensitivity, the ultraviolet sensitivity of the *rad14Δ* mutation is greatly enhanced when it is combined with the *rad52Δ* mutation, which presumably is due to the increased role of the *RAD52*



These results confirmed that the *RAD14* gene had been deleted. The construction of *rad1Δ::LEU2* and *rad6Δ::LEU2* deletions in strain DBY747 was done as described previously^{22,23}. Genomic deletions of the *RAD52* gene in strains DBY747 and DBY747 *rad14Δ::LEU2* were generated by transforming these strains with a 3.3-kb *BamHI* fragment of pSM22 in which the *BglII-Clal* fragment in the *RAD52* ORF had been replaced by the *URA3* gene. Replacement of the *RAD1* gene by the *URA3* gene in strain DBY747 *rad14Δ::LEU2* was done as described previously¹¹. The *RAD6* gene in strain DBY747 *rad14Δ::LEU2* was replaced by the *URA3* gene by transforming this strain with *BamHI-HindIII* digested plasmid pDG47 which contains the *URA3* gene flanked by *RAD6* nontranslated sequences. Deletion of each *RAD* gene was confirmed by testing allelism with known *rad* mutations.

recombination pathway in postreplication repair in the absence of excision repair¹⁴. These observations indicate that *RAD14* functions in the excision repair pathway. To investigate whether *RAD14* is essential for incision of ultraviolet-damaged DNA, the *rad14Δ* mutation was coupled to the temperature-sensitive DNA ligase mutant *cdc9-2*, and the incidence of incision breaks in DNA determined⁴. No incision breaks were formed in the *rad14Δ cdc9-2* cells, indicating a requirement of *RAD14* in the incision of ultraviolet-damaged DNA (data not shown). Thus, at least six genes, *RAD1*, *RAD2*, *RAD3*, *RAD4*, *RAD10* and *RAD14*, are indispensable for incision of ultraviolet-damaged DNA in yeast. Our observation that *RAD14* function is apparently limited to excision repair strongly suggests that the high incidence of skin cancers in XP-A patients is probably a consequence of defective excision repair rather than a deficiency in other cellular processes. □

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Signalling by the *sevenless* protein tyrosine kinase is mimicked by Ras1 activation

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CELL-FATE specification of R7 photoreceptors in the developing *Drosophila* eye depends on an inductive signal from neighbouring R8 cells. Mutations in three genes, *sevenless* (*sev*), *bride-of-sevenless* (*boss*) and *seven-in-absentia* (*sina*) cause the R7 precursor to become a non-neural cone cell¹⁻³. The *sev* gene encodes a receptor protein tyrosine kinase (Sev) localized on the R7 surface, activated by a *boss*-encoded ligand presented by R8 (refs 4-6). The *sina* gene encodes a nuclear factor required in R7 (ref. 3). Reduction in the dosage of the *Ras1* gene impairs Sev-mediated signalling, suggesting that activation of Ras1 may be an important consequence of Sev activation⁷. We report here that Ras1 activation may account for all of the signalling action of Sev; an activated Ras1^{Val12} protein rescues the normal R7 precursor from transformation into a cone cell in *sev* and *boss* null mutants and induces the formation of supernumerary R7 cells. Similar activation of the *Drosophila* Ras2 protein does not produce these effects, demonstrating Ras protein specificity.

To ask whether Ras1 activation alone is sufficient for Sev-mediated signalling, we used *sev* gene regulatory sequences to express wild-type and dominant activating *Ras1* alleles in those cells of the eye imaginal disc that normally express *sev*, R7, R3, R4, the cone cell precursors and the so-called 'mystery cells'^{5,8} (Fig. 1). During normal eye development, R3 and R4 are thought to be unable to respond to Sev because of earlier cell-fate commitment⁵, whereas the cone and mystery cells fail to adopt the R7 fate presumably because they do not contact R8, which alone expresses *boss*⁶. Ubiquitous expression of *boss* in the developing eye disc induces the cone cell precursors to differentiate as ectopic R7 cells⁹. Similarly, expression of a truncated, constitutively active Sev protein under *sev* gene control results in *boss*-independent rescue of the normal R7 cell and transformation of cone cell precursors and mystery cells into supernumerary R7 cells¹⁰. Identical results should thus be obtained with activated Ras1 under *sev* gene control if Ras1 activation can substitute for all of the Sev-mediated signal.

A copy of the wild-type *Ras1* gene expressed under *sev* gene control has no apparent effect on eye development; seven independent *P(sevRas1^{wt})* transgenic lines all have normal external and internal adult eye morphology and still require *sev* activity for R7 formation (Figs 2a and 3a, b, i). By contrast, 10 of 12 *P(sevRas1^{Val12})* lines bearing the dominant activating *Ras1^{Val12}* mutation¹¹ exhibit various degrees of external eye roughening (Fig. 2b, c). Adult eye sections of the less severely affected lines reveal that most ommatidia contain as many as four or five supernumerary R7 cells (Fig. 3c, j). Additional defects include aberrant packing and rotation of ommatidia, occasional extra and missing R1-6 cells, and missing pigment cells. In the more severely affected lines, these effects are

confined to peripheral eye regions with the central eye containing highly disorganized ommatidia completely devoid of pigment cells (Fig. 3d). The remaining two *P(sevRas1^{Val12})* lines appear wild type and represent insertions into inactive chromosomal sites, because mobilization of their inserts to new sites produces phenotypes similar to those described above (data not shown).

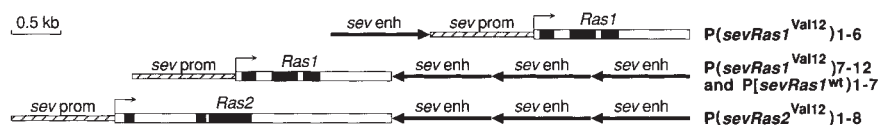
R7 cell formation in *P(sevRas1^{Val12})* flies does not require either *sev* or *boss* gene function. Small-rhabdomere R7-like cells were observed in all nine transgenic lines examined in a *sev^{d2}* null background and all six lines examined in a *boss¹* null background (Fig. 3e, f). To verify that these R7-like cells have properties of genuine R7 cells, we used a Rhodopsin 4 (*Rh4*)-*lacZ* promoter fusion that specifically labels the majority of terminally differentiated R7 cells¹². This marker labels a large number of cells in *sev^{d2}*; *P(sevRas1^{Val12})* retinæ and reveals that they project long axons to the proper R7 synaptic target layer of the medulla (Fig. 3m, n). The *sina* gene is still required for the production of R7 cells in all five *P(sevRas1^{Val12})* lines examined, indicating that Sina may act either downstream of Ras1 or in a separate pathway needed for R7 determination (Fig. 3g, h, o).

To demonstrate that Ras1^{Val12} bypasses the requirement for *sev* gene function in the normal R7 precursor and to identify the cells that are transformed into supernumerary R7 cells, eye discs were stained with an antibody specific to a neural antigen, the product of the *Drosophila elav* locus^{13,14}. *P(sevRas1^{wt})* discs display a wild-type pattern of R1-8 neural recruitment into developing ommatidia (Fig. 4a-d). *P(sevRas1^{Val12})* discs also exhibit normal R1-8 recruitment. Moreover, in *sev^{d2}*; *P(sevRas1^{Val12})* discs, the normal R7 precursor usually expresses the *elav* antigen, confirming that activated Ras1^{Val12} rescues the true R7 precursor from becoming a non-neural cone cell in *sev* (Fig. 4g). The neural antigen is also often expressed in one or both mystery cells and some or all of the four cone cell precursors present in each ommatidium (Fig. 4e-j).

We also generated transgenic flies bearing the equivalent activating allele of another Ras gene, *Ras2*, to investigate potential specificities of *Drosophila* Ras proteins in R7 cell determination (Fig. 1). The Ras2 protein is ~50% identical to Ras1 and is most closely related to the R-Ras family of Ras proteins^{15,16}. Eight *P(sevRas2^{Val12})* lines have identical weak rough eye phenotypes (Fig. 2d), consistent with the effects of this *Ras2^{Val12}* allele under endogenous or heat-inducible control¹⁷. Supernumerary R7 cells are not observed in *P(sevRas2^{Val12})*; instead, both inner and outer photoreceptors are occasionally missing and ommatidia are improperly oriented (Fig. 3k). In the disc, the *elav* antigen is not expressed in cone cell precursors or mystery cells (data not shown). Finally, virtually all ommatidia in *sev^{d2}*; *P(sevRas2^{Val12})* lack the R7 cell, although occasionally a few ommatidia in the centre of the eye contain an R7 (Fig. 3l, p). These rare *sev*-independent R7 cells may reflect a very weak ability of the overexpressed Ras2^{Val12} protein to contribute to the Sev-mediated signalling pathway. Nevertheless, our results indicate that Ras1 and not Ras2 is a normal target for the Sev-mediated signal, which is consistent with the isolation of mutations in *Ras1* but not in *Ras2* as enhancers of *sevenless*⁷.

The ability of Ras1^{Val12} to reproduce the activated Sev phenotype¹⁰ and to bypass normal requirements for *sev* and *boss* suggests that Ras1 activation may be the primary consequence of ligand-induced Sev signalling during R7 cell

FIG. 1 Structures of *sevRas* constructs used to produce transgenic *Drosophila*. The transformation vectors contain *sev* enhancer (*sev* enh) and *sev* promoter (*sev* prom) sequences from +7,134 to +7,833 base pairs (bp)²⁶ and -966 to +88 bp²⁷, respectively. The *Ras1* gene is a 1.6-kilobase (kb) *NruI-EcoRI* fragment¹⁶. The *Ras2^{Val12}* gene is a 2.8-kb *HpaI-XhoI* fragment¹⁷. Shaded portions represent protein coding regions. DNA manipulations and *Drosophila* transformation were done as in refs. 28 and 29, respectively.



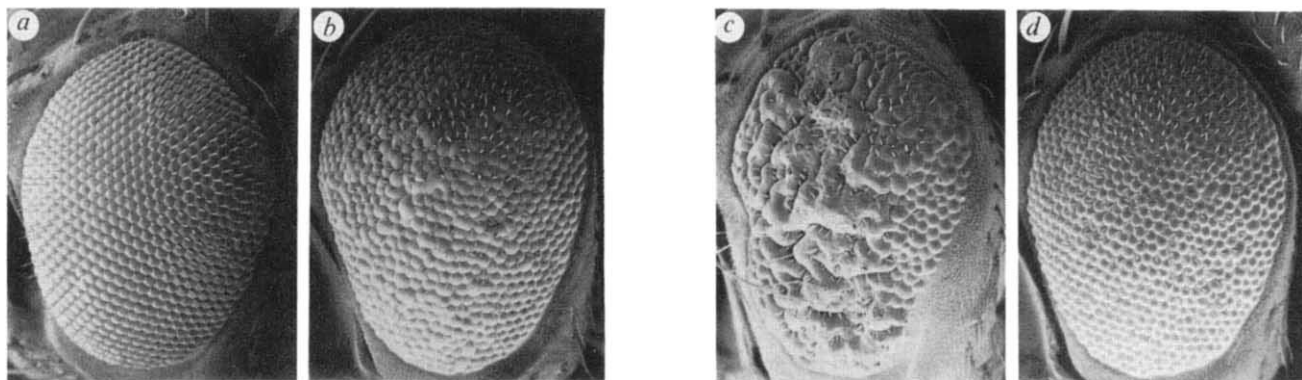


FIG. 2 Scanning electron micrographs (SEM) of eyes of $P(sevRas1^{wt})$ (a), a mildly rough $P(sevRas1^{Val12})$ line (b), a severely rough $P(sevRas1^{Val12})$ line

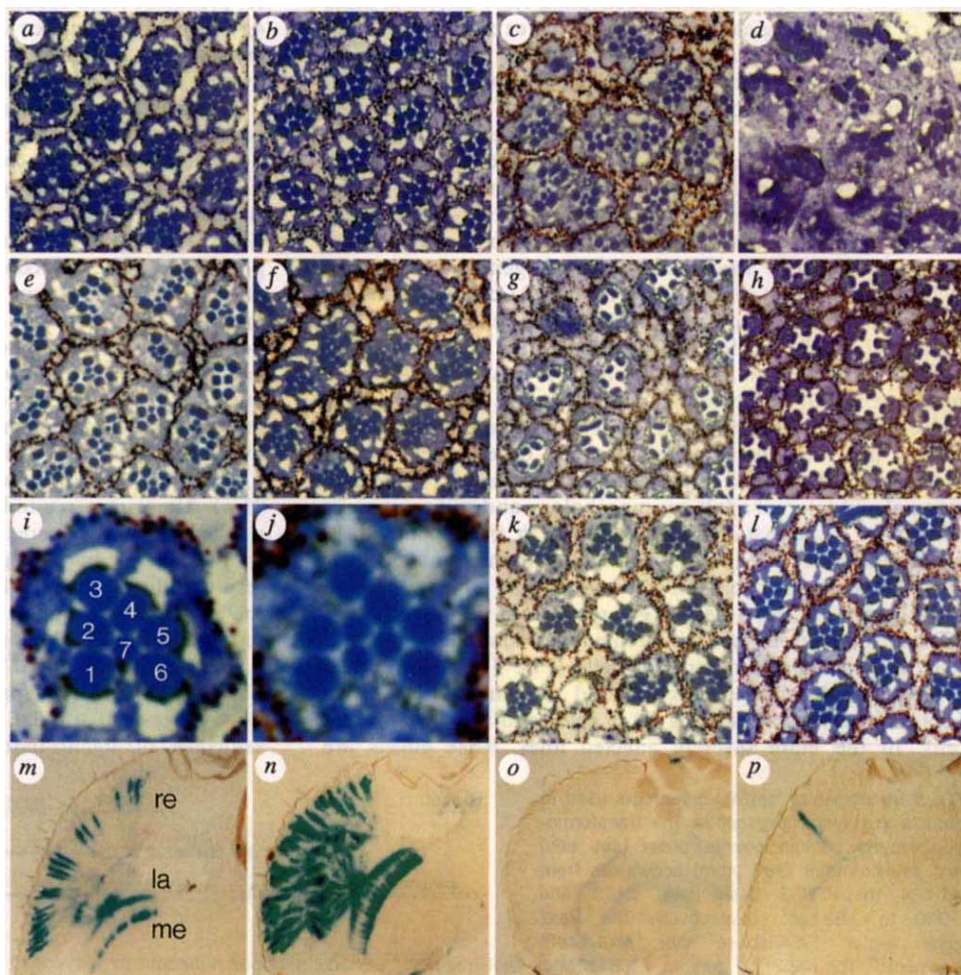
(c), and $P(sevRas2^{Val12})$ (d). Magnification $\times 100$; anterior at left, dorsal at top. SEM is described in ref. 30.

determination. But our results cannot exclude the possibility that endogenous Ras1 protein is not activated to the levels achieved by $Ras1^{Val12}$ and that normal Sev-mediated signalling requires additional pathways operating in parallel to the Ras1 pathway. Because Ras proteins have been implicated in a variety of signalling processes¹⁸, the consequences of Ras1 activation may be highly dependent on the cellular context. In this regard, the R3 and R4 photoreceptor precursors express *sev* but are not transformed into R7 cells by activated Sev¹⁰ or $Ras1^{Val12}$, perhaps because they are already committed to an alternative neural fate. One possibility is that Ras1 must be activated in all photoreceptor precursors for neural fate specification only and thus has already been activated in R3 and R4 by different means, perhaps

involving other protein tyrosine kinases. Indeed, *Ras1* is required for the development of all photoreceptors and interacts genetically with the locus encoding the *Drosophila* epidermal growth factor receptor homologue⁷.

Suppression of the *sev* phenotype by dominant activated $Ras1^{Val12}$ is strikingly similar to genetic interactions in *Caenorhabditis elegans* vulval development. Vulva formation requires the activities of a receptor protein tyrosine kinase and a Ras protein, the products of the *let-23* and *let-60* genes, respectively^{19,20}. Dominant activating mutations in *let-60* bypass the requirement for *let-23* and produce a multivulva phenotype²¹, perhaps equivalent to the supernumerary R7 phenotype caused by $P(sevRas1^{Val12})$. The pre-eminent role of Ras activation in

FIG. 3 Analysis of internal eye morphologies in *sevRas* transgenic flies. a–l, 1- μ m tangential plastic sections through the apical retina of $P(sevRas1^{wt})$ (a); $sev^{d2};P(sevRas1^{wt})$ (b); mildly rough $P(sevRas1^{Val12})$ (c); severely rough $P(sevRas1^{Val12})$ (d); $sev^{d2};P(sevRas1^{Val12})$ (e); $P(sevRas1^{Val12});boss^1$ (f); $P(sevRas1^{Val12});sina^{A16}$ (g); $sina^{A16}$, small rhabdomeres are apically extended R8 rhabdomeres³ (h); $P(sevRas1^{wt})$ single ommatidium, R cells are labelled as in ref. 31 (i); $P(sevRas1^{Val12})$ single ommatidium (j); $P(sevRas2^{Val12})$ (k); and $sev^{d2};P(sevRas2^{Val12})$ (l). m–p, 14- μ m horizontal cryostat sections stained for β -galactosidase activity of $P(Rh4.1900lacZ)$ in $P(sevRas1^{wt})$ (re, retina; la, lamina; me, medulla; anterior at top) (m); $sev^{d2};P(sevRas1^{Val12})$ (n); $P(sevRas1^{Val12});sina^{A16}$ (o); and $sev^{d2};P(sevRas2^{Val12})$ (p). Magnifications: a–h, k, l, $\times 1,100$; i, j, $\times 3,900$; m–p, $\times 120$. Plastic sectioning of adult eyes and histological staining of cryostat sections were done as in refs. 1 and 12, respectively.



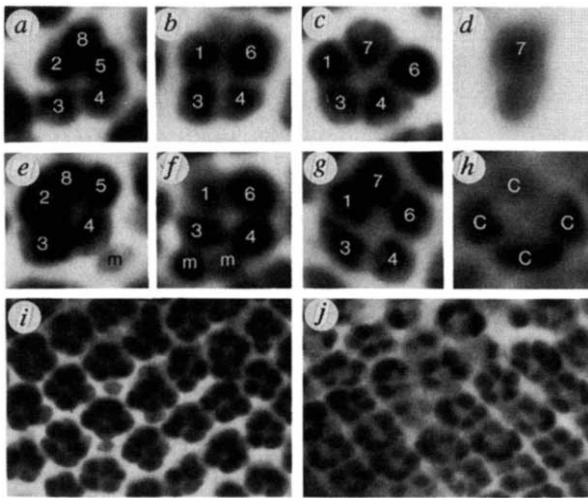


FIG. 4 Neural recruitment in *sevRas1* transgenic flies examined by anti-ELAV nuclear staining. *a-d*, Temporal sequence of single developing ommatidial clusters in *P(sevRas1^{Val12})* showing normal assembly of five-cell stage pre-clusters (*a*), addition of R1 and R6 (R2/R5/R8 nuclei remain in a lower focal plane) (*b*), addition of R7 (*c*), and absence of staining in cone cell precursor nuclei located just apical of the R7 nucleus (*d*). *e-h*, Equivalent sequence in *P(sevRas1^{Val12})* showing faint anti-ELAV staining of one mystery cell (*m*) at the five cell precluster stage (*e*), strong staining of both mystery cells in an ommatidial cluster at the time R1 and R6 are recruited (*f*), normal neural recruitment of R7 in a *sev^{d2};P(sevRas1^{Val12})* ommatidium (*g*), and anti-ELAV staining of cone cell precursors (*c*) (*h*). *i, j*, Overviews of anti-ELAV stained *P(sevRas1^{Val12})* disc regions showing that during early ommatidial assembly, about 50% of the preclusters contain one or two stained mystery cells (*i*) and that during later ommatidial development, a similar proportion display staining of up to four cone cell precursors (*j*). Magnifications: *a-h*, $\times 2,200$; *i, j*, $\times 1,000$. Eye disc antibody stainings were done as in ref. 30 using a 1:100 dilution of rat polyclonal anti-ELAV primary antibody followed by a 1:400 dilution of goat anti-rat HRP-conjugated IgG secondary antibody.

signalling by the structurally dissimilar *sev* and *let-23* receptor protein tyrosine kinases is surprising as mammalian tyrosine kinases are thought to act on a variety of downstream targets^{22,23}. Moreover, Ras activation alone cannot explain the many examples of a single cell type in which distinct physiological responses are elicited by stimulation of different tyrosine kinases^{24,25}. Nevertheless, our results and those obtained in *C. elegans* suggest that Ras activation may be a primary means by which protein tyrosine kinases exert their effects in many signal transduction pathways. □

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Lactose binding to heat-labile enterotoxin revealed by X-ray crystallography

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RECOGNITION of the oligosaccharide portion of ganglioside G_{M1} in membranes of target cells by the heat-labile enterotoxin from *Escherichia coli* is the crucial first step in its pathogenesis, as it is for the closely related cholera toxin^{1–3}. These toxins have five B subunits, which are essential for G_{M1} binding, and a single A subunit, which needs to be nicked by proteolysis and reduced, yielding an A1-‘enzyme’ and an A2-‘linker’ peptide. A1 is translocated across the membrane of intestinal epithelial cells, possibly after endocytosis^{4,5}, upon which it ADP-ribosylates the G protein $G_{s\alpha}$ (reviewed in refs 2, 3, 6). The mechanism of binding and translocation of these toxins has been extensively investigated^{1,2,7–20}, but how the protein is orientated on binding is still not clear^{10–12,18}. Knowing the precise arrangement of the ganglioside binding sites of the toxins will be useful for designing drugs against the diarrhoeal diseases caused by organisms secreting these toxins and in the development of oral vaccines against them^{21,22}. We present here the three-dimensional structure of the *E. coli* heat-labile enterotoxin complexed with lactose. This reveals the location of the binding site of the terminal galactose of G_{M1} , which is consistent with toxin binding to the target cell with its A1 fragment pointing away from the membrane. A small helix is identified at the carboxy terminus of A2 which emerges through the central pore of the B subunits and probably comes into contact with the membrane upon binding, whereas the A1 subunit is flexible with respect to the B pentamer.

The three-dimensional structure of heat-labile enterotoxin (LT) complexed with lactose (Gal- β 1-4-Glc) was determined by X-ray crystallography at 2.3 Å resolution (Fig. 1). Lactose is found to bind virtually identically to all five B subunits (Table 1; Fig. 2), interacting mainly through its galactose moiety. Hence the sugar binding site is identified as the terminal galactose in G_{M1} (which is Gal- β 1-3-GalNAc- β 1-(NeuAc- α 2-3)-4Gal- β 1-4-Glc cerebroside) for steric reasons—the numerous saccharide-protein interactions at this binding site will not allow any substituents other than at position C1. This agrees with earlier observations that the terminal galactose is important in G_{M1} binding^{7,8,13,16,17}. The glucose moiety bound to galactose in lactose is replaced in G_{M1} by a β 1-3-linked *N*-acetyl-galactosamine.

The specificity of the galactose binding stems from the fact that every hydrogen-bond donor and acceptor of galactose, except the ether oxygen and the O1 linker to glucose, is engaged in hydrogen bonding. These involve the side chains of residues

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Glu 51, Gln 61, Asn 90 and Lys 91, as well as the carbonyl oxygen of residue 56 and three well defined water molecules (Table 1; Fig. 2). In agreement with earlier work^{9,14}, there are extensive van der Waals contacts between hydrophobic groups of galactose and Trp 88 (Fig. 2a), a type of interaction observed in other sugar-binding proteins^{23,24}. His 57 is also in van der Waals contact with the C6 atom of galactose. Lys 91 is also involved, which agrees with the key role proposed for lysine residues in G_{M1} binding¹⁴. As more than 80% of the amino-acid sequence of LT is identical in cholera toxin⁶, it is no surprise that all residues directly interacting with galactose are conserved in cholera toxin. Differences occur only in the second shell (Fig. 2b). The difference at position 95 might explain why LT recognizes certain glycoproteins besides G_{M1} (presumably galactoproteins)^{8,13,16} when cholera toxin does not, although there could be further differences in the binding sites of the other sugar residues of G_{M1} .

FIG. 3 a, Stereo view of heat-labile enterotoxin ($C\alpha$ tracing) with lactose bound to all five B subunits. Lactoses are shown in green, binding far from the A subunit (yellow) with galactose buried in a small depression in the B subunits (purple), and glucose reaching out, away from the A1 subunit. As discussed in the text, the position and orientation of the galactose unit suggest that the AB_5 molecule binds with the 'convoluted' surface of the B pentamer towards the membrane and A subunit away from the membrane. In addition the $C\alpha$ trace of the A subunit of the native LT structure is shown in orange after superposition of its B pentamer on LT-lactose, illustrating the change in relative orientation of 5° of the A1 fragment with respect to the B pentamer. Many atoms of the A subunit, particularly those located far from the B pentamer where the active site is situated²⁵, are shifted by more than 3 Å between the two structures.

b, Ribbon plot³⁵ showing the remarkable conformation of the LT A subunit. Note the small helix at the bottom of the figure at the C terminus of A2. Residues 189–195 are missing in the top left corner of the figure as they are not clear in density like the two C-terminal residues of A2. c, Ribbon plot showing the AB_5 complex of LT; the A2 subunit is shown in orange to emphasize the small helix at the C terminus of this subunit, which reaches the surface of the B pentamers on the 'convoluted' surface, explaining how it is possible to engineer a 53-residue protein at this C terminus without disturbing AB_5 complex formation³⁶.

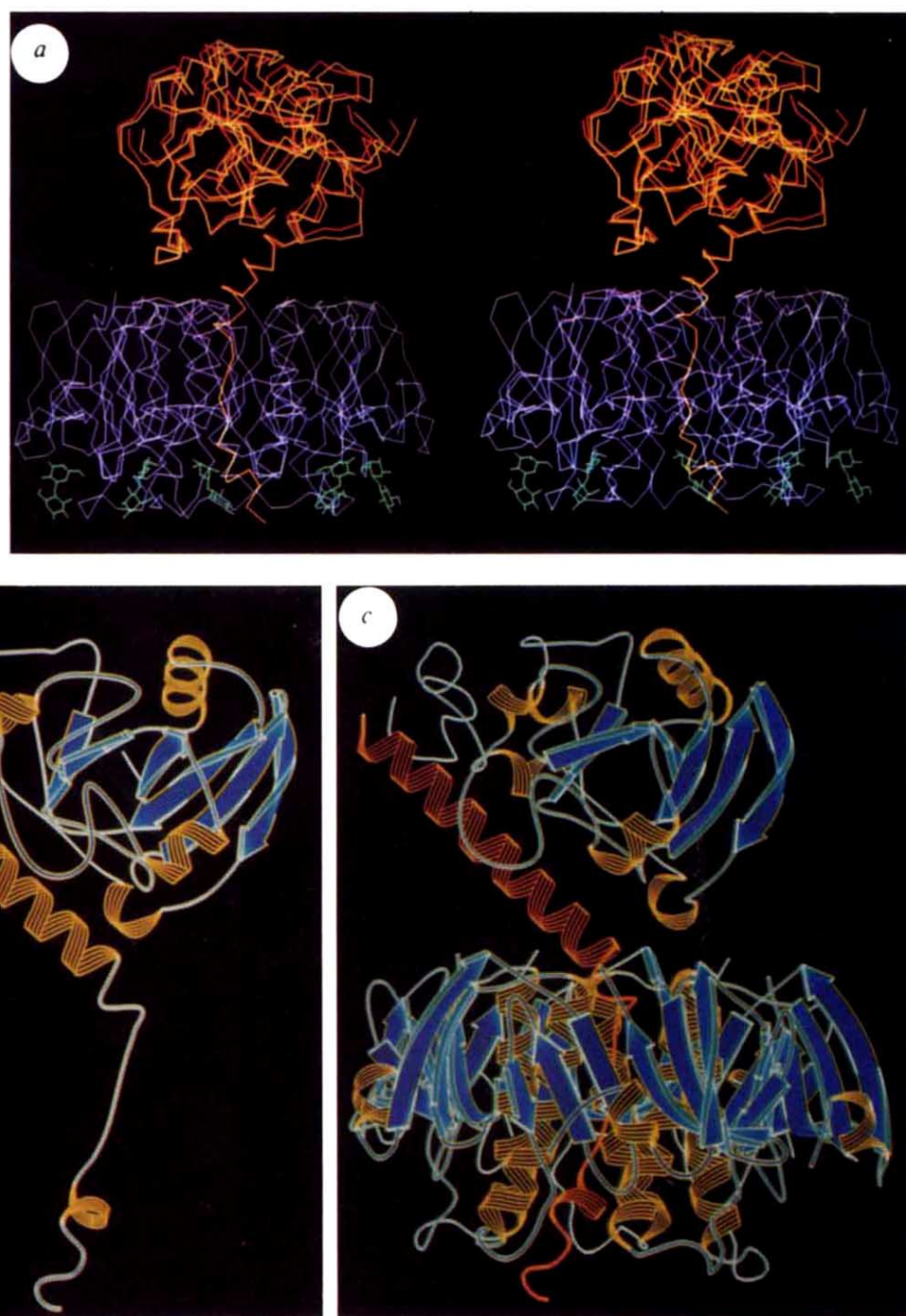


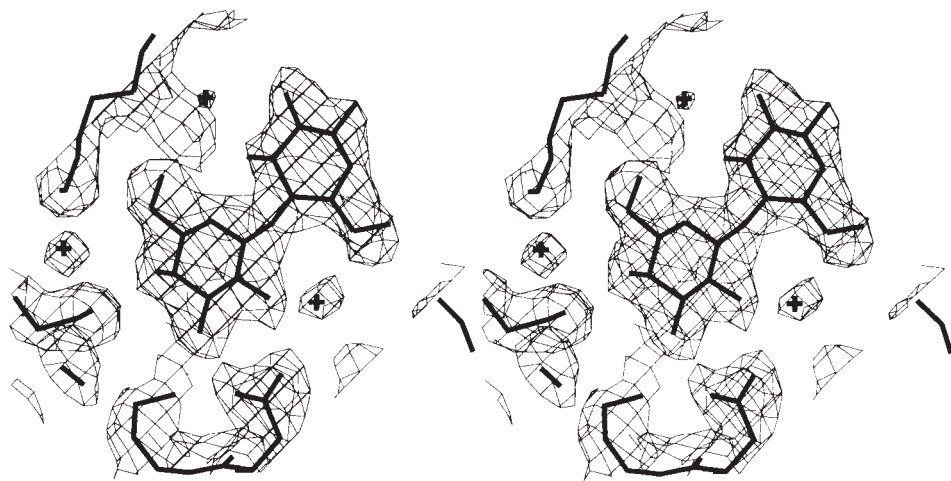
TABLE 1 Galactose hydrogen bonding

Galactose	Atom	Ligands	No.	Hydrogen bonds to B subunits				
				B(1)	B(2)	B(3)	B(4)	B(5)
O ₂	O	Water	A	2.8	3.0	3.1	3.1	—
	N ^B ₂	Asn	90	3.0	2.8	2.8	3.2	2.9
O ₃	N ^B ₁	Lys	91	2.9	2.9	2.7	2.9	3.0
	O ^B ₁	Asn	90	2.9	2.7	3.2	2.7	2.8
O ₄	O ^B ₂	Glu	51	2.7	2.8	2.6	2.7	2.6
	N ^B ₁	Lys	91	3.0	2.9	3.0	3.0	2.8
O	O ^B ₁	Water	B	2.9	3.2	2.8	3.1	2.9
	N ^B ₂	Gln	61	3.0	3.0	3.1	3.0	2.8
O	O	Gln	56	3.0	3.0	3.0	3.0	3.4
	O	Water	C	—	3.2	3.0	3.2	—

Hydrogen-bond distances, in ångströms, between the galactose moiety and the B pentamer of LT in the LT-lactose complex. B subunits are numbered according to ref. 25. Differences between the subunits reflect the uncertainty in the coordinate positions, estimated at 0.2 Å (ref. 29), as well as some slight differences induced by crystal contacts. Waters are indicated as A, B, C to emphasize the different water positions.

FIG. 1 Electron density of lactose (SIG-MAA weighted $2m|F_o|-D|F_c|$, phases α_c (ref. 29)) in subunit B(2), contoured at the 1σ level between 15–2.3 Å. The five glucose moieties are ill defined, with mean temperature factor values of 56–70 Å², except in subunit B(4), where the glucose takes part in a crystal contact. Mean temperature factor values for galactose are 23 Å² in subunits B(1), B(2) and B(4) and 40 Å² in subunit B(3) and B(5).

METHODS. Co-crystals of the porcine variant of LT, which differs by less than 3% in sequence from human LT³⁰, and lactose were grown by three-layer liquid-liquid diffusion in a modification of the native two-layer crystallization procedure³¹: a middle layer was added to a final concentration of 25 mM lactose and potassium fluoride was replaced by NaCl. Crystals in spacegroup $P2_12_12_1$ had cell dimensions of 119.8, 101.2, 64.2 Å, which are similar to, but significantly different from, the native values of 119.2, 98.2 and 64.8 Å. Data were collected on an Enraf Nonius FAST area detector



with an Elliot GX21 rotating anode, to a resolution of 2.3 Å with a completeness of 91% ($F > 1\sigma$) and an R_{sym} of 6.4%. The structure was solved by molecular replacement followed by rigid body and coordinate refinement in

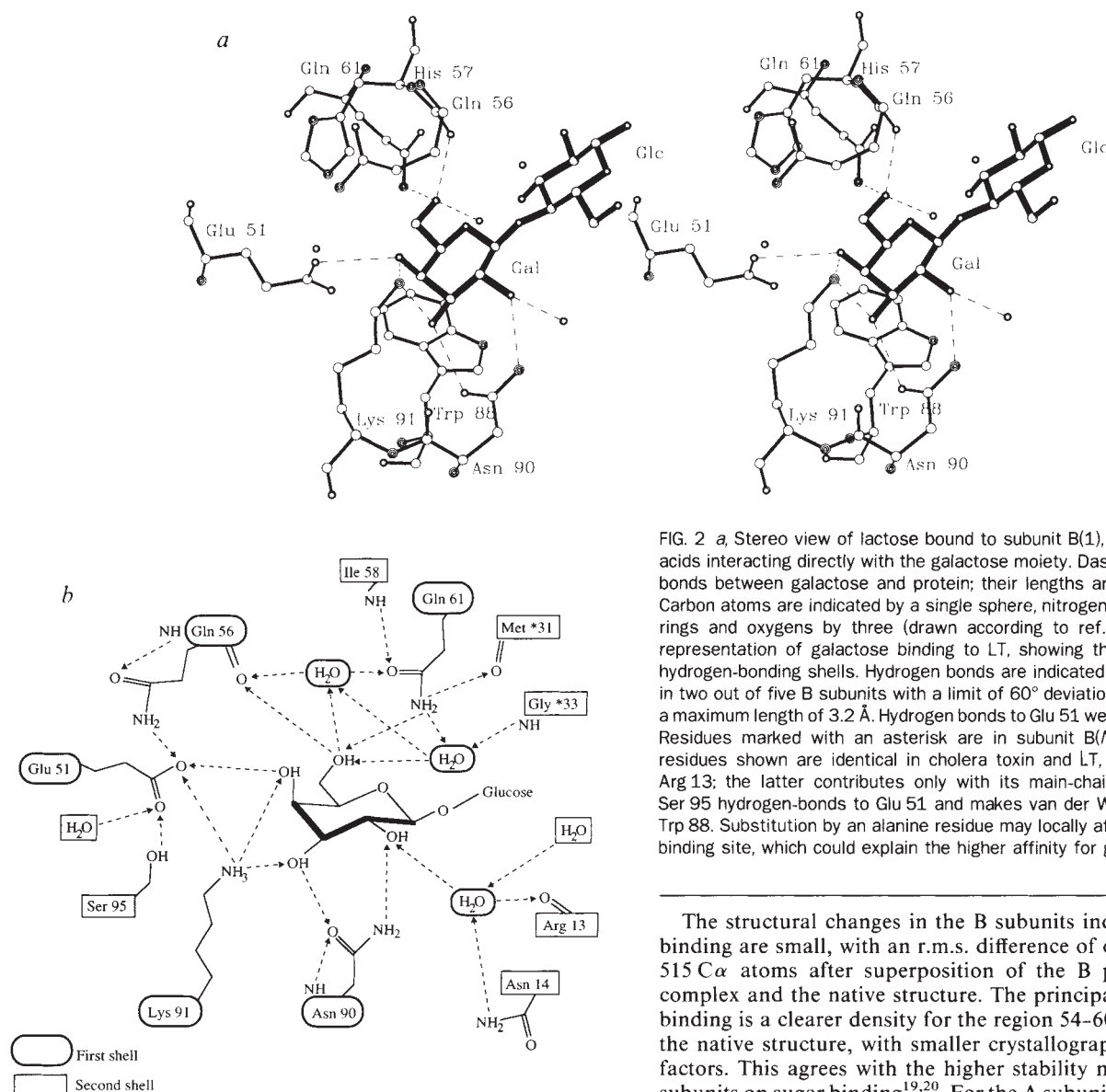


FIG. 2 *a* Stereo view of lactose bound to subunit B(1), showing the amino acids interacting directly with the galactose moiety. Dashed lines, hydrogen bonds between galactose and protein; their lengths are given in Table 1. Carbon atoms are indicated by a single sphere, nitrogens by two concentric rings and oxygens by three (drawn according to ref. 34). *b*, Schematic representation of galactose binding to LT, showing the first and second hydrogen-bonding shells. Hydrogen bonds are indicated only if they appear in two out of five B subunits with a limit of 60° deviation from linearity and a maximum length of 3.2 Å. Hydrogen bonds to Glu 51 were less well defined. Residues marked with an asterisk are in subunit B($N+1$) (ref. 25). All residues shown are identical in cholera toxin and LT, except Ser 95 and Arg 13; the latter contributes only with its main-chain carbonyl oxygen. Ser 95 hydrogen-bonds to Glu 51 and makes van der Waals contacts with Trp 88. Substitution by an alanine residue may locally affect the galactose-binding site, which could explain the higher affinity for galactose of LT⁸.

The structural changes in the B subunits induced by lactose binding are small, with an r.m.s. difference of only 0.4 Å on all 515 C α atoms after superposition of the B pentamer of the complex and the native structure. The principal effect of sugar binding is a clearer density for the region 54–60 compared with the native structure, with smaller crystallographic temperature factors. This agrees with the higher stability noticed for the B subunits on sugar binding^{19,20}. For the A subunit, however, there

is distinct rigid body movement with respect to the B pentamer. Residues A:1-222 (that is, the A1 fragment plus the long N-terminal helix of A2) move by a 5° rotation, with residues 216-222 functioning as a hinge (Fig. 3a). This type of rotation is also found in other crystal forms of LT without bound sugars (our unpublished results), but the remarkable flexibility may be important for translocation of LT and cholera toxin across the membrane.

One unexpected result was the well-defined density for five C-terminal residues of the A2 fragment (comprising residues A:196-240), which were poorly defined in the native map at 2.3-Å resolution²⁵. In the LT-lactose complex, residues A2:232-235 adopt a helical conformation (Fig. 3b). Hence the unusual A2 fragment consists of (1) a long N-terminal helix, interacting with the A1 enzyme; and (2) an extended chain, comprising residues 227-231, protruding through the central pore of the B pentamer along the 5-fold axis; and (3) a one-and-a-half turn C-terminal helix (Fig. 3c). Temperature factors are relatively high for residues 232-235 (on average 45 Å² versus an average of 27 Å² for the entire A2, and 18 Å² for the B pentamer) and hence this helix may be poised for conformational change. The C-terminal helix is also found in a higher-resolution native LT structure based on 1.95 Å data (T.K.S. *et al.*, manuscript in preparation) and therefore seems to be an intrinsic feature of LT.

The orientation of the bound galactose (Fig. 3a) is such that the C1 linker atom is in the middle of the 'convoluted' surface of the B pentamer, about 8 Å horizontally away from the side of the pentamer and at least 25 Å vertically away from the opposite 'flat' or 'A-binding' surface. This distance is too far for the remaining sugar residues of the G_{M1} oligosaccharide to span because they extend 25 Å at most from the hydrophobic part of the membrane²⁶. Binding with the A subunit facing the membrane would not only force the A subunit into the membrane but also insert the hydrophilic B pentamer into the hydrophobic part of the membrane by at least 8 Å, in contradiction to biochemical data^{10,11,18}. As there is little or no evidence for any large conformational change in the B pentamer upon G_{M1} binding^{9,14,20}, the initial binding of LT or cholera toxin to G_{M1}-containing membranes most probably occurs with the A subunit pointing away from the membrane, when all five ganglioside binding sites are occupied. This mode of binding has already been proposed^{5,10} but others have come to different conclusions^{11,12,18}. It should be noted that here the C terminus of A2 interacts with the membrane (Fig. 3), in good agreement with photoaffinity labelling studies¹⁰. The C-terminal sequence of A2 (ArgAspGluLeu) closely resembles the LysAspGluLeu sequence in cholera toxin; this KDEL sequence may act as a retention signal in the endoplasmic reticulum membrane²⁷ and could be important for the interaction of both toxins with the cell membrane.

Finally, it has been pointed out to us that the topology of monomeric *Staphylococcus aureus* nuclease²⁸ is essentially the same as that of the B subunit of LT, although the relative orientation of the secondary-structure elements is different (A. Muzzin, personal communication). But the position of the galactose-binding site described here corresponds roughly with the active site of the nuclease, which may be of interest from the point of view of evolution in that LT, cholera toxin and the nuclease are all secreted by bacterial pathogens. □

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Selection of single-stranded DNA molecules that bind and inhibit human thrombin

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APTAMERS¹ are double-stranded DNA or single-stranded RNA molecules that bind specific molecular targets. Large randomly generated populations can be enriched in aptamers by *in vitro* selection and polymerase chain reaction¹⁻¹¹. But so far single-stranded DNA has not been investigated for aptamer properties, nor has a target protein been considered that does not interact physiologically with nucleic acid. Here we describe the isolation of single-stranded DNA aptamers to the protease thrombin of the blood coagulation cascade and report binding affinities in the range 25-200 nM. Sequence data from 32 thrombin aptamers, selected from a pool of DNA containing 60 nucleotides of random sequence, displayed a highly conserved 14-17-base region. Several of these aptamers at nanomolar concentrations inhibited thrombin-catalysed fibrin-clot formation *in vitro* using either purified fibrinogen or human plasma.

We synthesized a pool of ~10¹³ 96-mer oligodeoxyribonucleotides that share 18-nucleotide binding sites for polymerase chain reaction (PCR) primers at their 5' and 3' termini and also contain 60-nucleotide randomly generated sequences. Using a 5'-biotinylated primer for one strand, this pool was amplified and radio-labelled by PCR. The nonbiotinylated strand was isolated from its complementary strand after application to an avidin-agarose column and base denaturation¹². Single-stranded DNA was then applied to a concanavalin A (con A)-agarose column to remove DNA with affinity for con A, and the eluent applied to human thrombin immobilized on con A. After washing unbound DNA from the column, thrombin-DNA complexes were eluted with a con A ligand, α -methylmannoside. Fractions containing thrombin were identified using a chromogenic substrate assay and DNA from those fractions was quantitated before PCR

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TABLE 1 Sequence specificity of thrombin inhibition

Sample	Sequence	Concentration	Clotting time (s)	
			Purified fibrinogen	Human plasma
No DNA	—	—	25 ± 1	25 ± 1
Clone 29	96-mer	100 nM	76 ± 3	50 ± 2
Unselected DNA	96-mer	100 nM	26 ± 1	26 ± 1
Consensus 15-mer	GGTGGTGTGGTTGG	100 nM	169 ± 8	43 ± 2
Scrambled 15-mer	GGTGGTGGTGTGGT	100 nM	26 ± 1	26 ± 1
Consensus 6-mer	GGTTGG	20 μM	42 ± 2	40 ± 2
Scrambled 6-mer	TGGGGT	20 μM	26 ± 1	26 ± 1

DNA was incubated for 1 min at 37 °C in either selection buffer (0.2ml; Fig. 1 legend) containing human fibrinogen (Sigma) or fresh human plasma. Thrombin (0.1 ml in selection buffer pre-equilibrated to 37 °C) was added to give a final concentration of 13 nM thrombin and the indicated concentrations of DNA; the final concentration of purified fibrinogen was 2 mg ml⁻¹. Clotting times were measured using an automated fibrometer. Human blood was obtained by venipuncture, anticoagulated with the addition of 0.1 volumes 3.8% sodium citrate and fractionated by centrifugation (at 2,000g for 5 min). Plasma was decanted and stored (for less than one day) at 4 °C. Clotting times are the average of three experiments.

amplification and repeated selection. The scheme for the selection and amplification cycle is shown in Fig. 1.

Only 0.01% of the input DNA eluted with thrombin during the first selection cycle; this percentage increased in subsequent rounds of selection to ~40% by selection cycle 5. DNA from selection cycle 5 was assayed for thrombin binding specificity on nitrocellulose filters. The results showed that a significant fraction (~30%) of aptamer DNA bound to thrombin but not to either ovalbumin or human fibrinogen (data not shown). Cycle-5 DNA was cloned in order to test the affinity of individual aptamers for thrombin, and radiolabelled single-stranded DNA from several clones was used to measure dissociation constants (K_d). Several clones gave $K_d \approx 200$ nM, whereas the original pool of DNA showed little affinity for thrombin (data not shown).

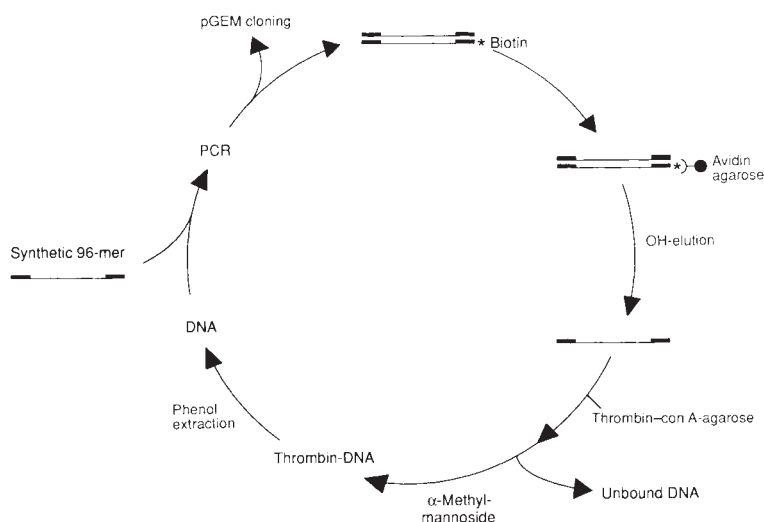
We determined the DNA sequence of the 60-nucleotide randomly generated region from 32 clones to examine both the heterogeneity of the selected population and to identify homologous sequences. Sequence analysis showed each of the 32 clones to be distinct, but there was a striking sequence conservation evident in every clone. The hexamer GGTTGG was found at a variable location within the 60-nucleotide randomized region in 31 out of 32 clones. The remaining clone contained the sequence TGTGG, a match of five out of six bases. Additionally, in 28 of the 32 clones, a second hexamer GGNTGG was located 2–5 nucleotides 5' or 3' from the hexamer

GGTTGG. The DNA sequence from the region of conservation from all 32 clones is presented in Fig. 2. Except for four clones that contain a close variation, the sequence GGNTGGN₂₋₅GGNTGG is conserved. A compilation of the data relating base frequency to position is also shown in addition to the derived consensus sequence. DNA sequencing of clones (>20) from the unselected DNA population or from a population of aptamers selected for binding to a different target revealed no homology to the thrombin-selected aptamers (data not shown). These results indicate that this consensus sequence is responsible, either wholly or in part, for conferring thrombin affinity on the 96-mer oligodeoxynucleotide.

We next analysed whether the aptamer DNA could inhibit the thrombin-catalysed conversion of fibrinogen to fibrin using either purified human fibrinogen or adult human plasma. Using clone 29 DNA (Fig. 2) and purified fibrinogen, fibrin formation was detected at 76 seconds, compared with 26 s with unselected DNA or 25 s in the absence of DNA (Table 1). More impressively, with respect to oligonucleotide size and potency, fibrin formation using the 15-mer GGTTGGTGTGGTTGG (contained in clones 15 and 29) was detected at 169 s. The 6-mer GGTTGG also inhibited thrombin, although a higher concentration was required. No inhibition was seen with 15-mer or 6-mer scrambled sequence controls. The aptamers had anti-clotting activity in human plasma, although they were less potent (Table 1). This difference in potency may be attributable to nuclease

FIG. 1 Scheme for the selection of DNA aptamers to human thrombin.

METHODS. 96-mer DNA was prepared by solid-phase phosphoramidite chemistry on a Biosearch 8600 synthesizer using an equimolar mixture of the four bases for the 60-nucleotide random portion of the sequence which is flanked by defined 18-nucleotide regions that allow for PCR priming by the following oligonucleotides: 5'-CGTACGGTCGACGCTAGC-3' and 5' biotin-GGATCCGAGCTCCACGTG-3' (biotinylation reagent from NEN). The synthetic DNA was purified by PAGE and amplified 100-fold by large-scale PCR to generate a sequence library with a complexity $>10^{13}$ individual sequences¹. DNA from this library was then amplified and radiolabelled by PCR under standard conditions in the presence of 60 μ Ci [α -³²P]dNTPs. The resulting biotinylated (asterisk) double-stranded DNA was applied to an avidin-agarose (Vector Labs) column ($\bullet$) equilibrated with 0.1M Tris-HCl, pH 7.5, 0.1M NaCl. ssDNA was then eluted in 0.15N NaOH (ref. 12). The sample was neutralized with acetic acid, concentrated, and the DNA precipitated with ethanol. Following centrifugation, DNA was redissolved in selection buffer (20 mM Tris-acetate, pH 7.4, 140 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂) and 100 pmol applied to 1 ml concanavalin A-agarose (Vector Labs) equilibrated in selection buffer. The flow-through was applied to 6 nmol of human thrombin (Sigma) immobilized on 1 ml concanavalin A-agarose equilibrated in selection buffer. After washing with several column volumes of selection buffer, thrombin-DNA complexes were eluted by the addition of 0.1M α -methylmannoside to the selection buffer wash. Fractions containing thrombin were identified using a chromogenic substrate assay (KabiVitrum), extracted with phenol, and the DNA precipitated by addition of 20 μ g glycogen and three volumes of ethanol. DNA was



pelleted by centrifugation, redissolved, and subjected to further cycles of amplification and selection. After the first selection cycle, DNA was amplified by PCR using the primers 5'-CGTACGGTCGACGCTAGC-3' and 5'-TAATAC-GACTCACTATAGGGATCCGAGCTCCACGTG-3', and subsequently cloned using the plasmid pGEM3Z (Promega). Single-stranded 96-mer DNA was then prepared from the clones using the primers 5' biotin-GGATCCGAGCTC-CACGTG-3' and 5'-CTGCAGGTCGACGCTAGC-3'.

Clone																								
1	g	G	G	t	T	G	G	-	-	g	t	c	G	G	t	T	G	G	t					
2	g	G	G	a	T	G	G	-	-	t	t	t	G	G	t	T	G	G	g					
3	a	G	G	t	T	G	G	-	-	g	a	G	G	g	T	G	G	g						
4	t	G	G	t	T	G	G	-	-	c	g	a	G	G	a	T	G	G	a					
5	a	G	G	t	T	G	G	-	-	g	t	a	g	t	G	t	T	G	G	t				
6	a	G	G	t	T	G	G	-	-	g	c	t	G	G	t	T	G	G	g					
7	g	G	G	t	T	G	G	-	-	g	a	G	G	t	T	G	G	a						
8	t	G	G	t	T	G	G	-	-	g	t	c	G	G	t	T	G	G	g					
9	g	G	G	a	T	G	G	-	-	t	g	t	G	G	t	T	G	G	c					
10	t	G	G	t	T	G	G	-	-	c	a	g	G	G	a	T	G	G	g					
11	t	G	G	a	T	G	G	-	-	t	g	a	G	G	t	T	G	G	a					
12	g	G	G	g	T	G	G	-	-	t	t	a	G	G	t	T	G	G	t					
13	a	G	G	g	T	G	G	-	-	t	t	a	G	G	t	T	G	G	t					
14	c	G	G	t	T	G	G	-	-	g	t	t	g	G	G	a	T	G	G	a				
15	c	G	G	t	T	G	G	-	-	t	g	t	G	G	t	T	G	G	t					
16	a	G	G	t	T	G	G	-	-	t	g	t	G	G	g	T	G	G	g					
17	c	G	G	g	T	G	G	-	-	a	t	a	G	G	t	T	G	G	a					
18	g	G	t	g	T	G	G	t	a	g	t	t	t	G	t	T	G	G	g					
19	t	G	G	t	T	G	G	t	t	a	c	t	G	G	t	T	G	G	g					
20	g	G	G	t	T	G	G	-	-	t	c	t	G	G	g	T	G	G	a					
21	t	G	G	t	T	G	G	-	-	g	t	t	G	G	g	T	G	G	a					
22	t	G	G	t	T	G	G	-	-	c	c	a	G	G	t	T	G	G	a					
23	c	t	a	g	c	G	G	-	-	c	a	g	t	G	G	t	T	G	G	g				
24	t	G	G	g	T	G	G	-	-	g	g	a	G	G	t	T	G	G	t					
25	a	G	G	t	T	G	G	-	-	t	t	t	G	G	g	T	G	G	t					
26	a	G	G	t	T	G	G	-	-	t	a	g	G	G	t	T	G	G	t					
27	g	G	G	a	T	G	c	-	-	g	g	t	G	G	t	T	G	G	g					
28	t	G	G	t	T	G	G	-	-	t	t	a	t	G	G	t	T	G	G	t				
29	a	G	G	t	T	G	G	-	-	t	g	t	G	G	t	T	G	G	c					
30	a	G	G	t	T	G	G	-	-	t	g	t	G	G	g	T	G	G	g					
31	t	G	G	t	T	G	G	-	-	g	a	G	G	t	T	G	G	t						
32	g	G	G	t	T	G	G	t	g	g	g	t	G	G	a	T	G	G	t					
Consensus sequence																								
G																								
A																								
T																								
C																								
(N) 3																								

FIG. 2 Thrombin aptamer sequence homology. DNA from the fifth cycle of thrombin selection was amplified by PCR and cloned (see Fig. 1 legend). The sequences of the randomly generated 60-mer inserts were determined for 32 clones using dideoxynucleotide chain termination. A region of homology was identified shown in bold upper case. Below is a tabulation of the data and the deduced consensus sequence.

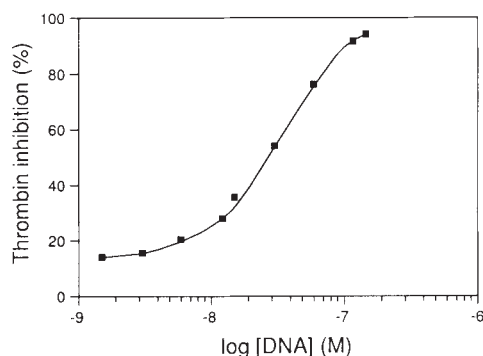


FIG. 3 Per cent thrombin inhibition versus DNA concentration. METHODS. Selection buffer containing human fibrinogen (2mg ml⁻¹ final) and varying concentrations of DNA (~1 nM–150 nM) was incubated for 1 min at 37 °C before adding thrombin as described in Table 1 legend. Clotting times were measured using an automated fibrometer. The extent of thrombin inhibition was then calculated using a thrombin standard curve generated by measuring clotting time versus thrombin concentration.

action or to binding of prothrombin or other plasma proteins present in much higher concentrations than thrombin. When the 6-mer was tested at much higher concentrations it was still as potent, perhaps because plasma nucleases and/or binding proteins were saturated. Thus, this protocol enabled us to isolate single-stranded DNA molecules that inhibit catalysis by thrombin. Moreover, this activity is retained by a short consensus sequence derived from the selected 96-mers.

We next determined the extent of thrombin inhibition with varying DNA concentration and found that thrombin was inhibited by 50% at 25 nM (Fig. 3). This is not a true inhibition constant, but it demonstrates the potency of the thrombin aptamer. Also, the data indicate that stoichiometry could be 1:1 and that each bound thrombin is largely, if not completely, inhibited.

Our findings show that a population of ~10¹³ molecules of 96-mer single-stranded DNA can be selected for aptamers that bind human thrombin, a protein with no known nucleic acid-binding function. As thrombin is a glycoprotein, we have been able to use lectin-agarose to immobilize it and α -methylmannoside to elute the thrombin-DNA complexes. Initially we bound DNA to thrombin that was covalently linked to agarose. Denaturing elution with EDTA gave single-stranded DNA with affinity for the matrix and hence only a modest enrichment of thrombin aptamers. These results suggest that the conditions under which aptamers are eluted from a covalently bound target are crucial to the successful isolation of high-affinity aptamers. One way to circumvent these problems may be to elute aptamer-target complexes from the matrix. Once isolated from matrix-associated oligonucleotides, the complexes can be fully denatured and the aptamers with the highest affinity recovered. In view of our success in isolating high-affinity aptamers to thrombin, we believe that lectin immobilization may provide an aptamer selection technique applicable to a large variety of glycoproteins.

The basic nature of thrombin may also have facilitated the isolation of aptamers; apart from the catalytic site, thrombin also contains an anion-binding exosite which binds fibrinogen^{13–15}, and a binding site for heparin, a polyanion. *In vitro* selection should allow the isolation of aptamers to most proteins, although the affinity may be highest for proteins rich in basic residues. The strong sequence dependence shown in Fig. 3 and in Table 1 suggests, however, that electrostatic interactions may be necessary but not sufficient for high-affinity thrombin binding.

It may be that the affinity of an aptamer for its ligand is comparable to that of an antibody for its antigen. The chemical nature, size and mode of isolation of aptamers may sometimes offer advantages over existing antibody technology. We are at present investigating the aptamer-binding site on thrombin and analysing the binding sequences of individual aptamers in an effort to understand the base relationships that mediate binding and inhibition, our long-term interest being to develop diagnostics and therapeutic agents. □

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The structure of the *E. coli* *recA* protein monomer and polymer

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Nature **355**, 318–325 (1992).

DUE to a production error after the return of corrected proofs, Figs 3*a* and 6*a* of this article were printed upside down. Correct orientations are shown here with relevant parts of the figure legends. Discussions in the text relating to the polymer structure and its relative polarity apply to the orientations shown here. Additionally, Figs 2, 3*b*, 4 and 5 are described relative to this orientation of Fig. 3*a*, and Fig. 6*b* shows an enlargement of the polymer contact region of the properly oriented Fig. 6*a*.

FIG. 6 Packing between filaments in the crystal. *a*, An α -carbon backbone representation of two turns each of two filaments are shown in yellow and blue, ADP in red. The boxed region shows the interfilament contact region enlarged in *b*.

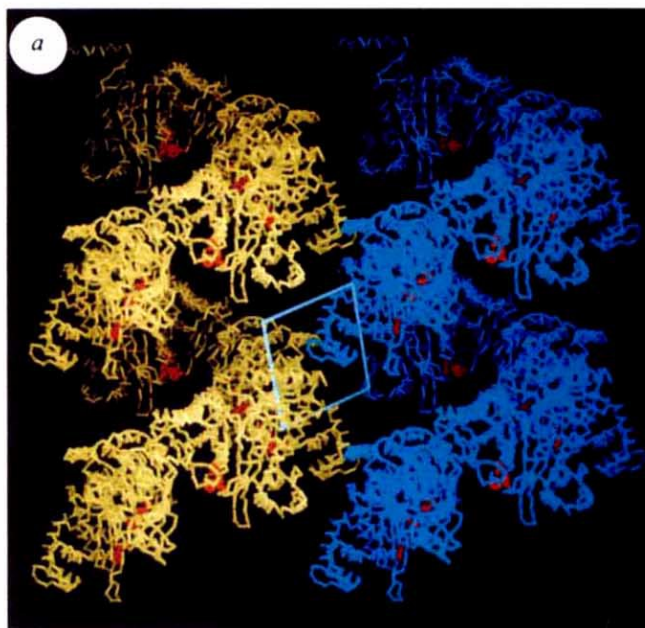
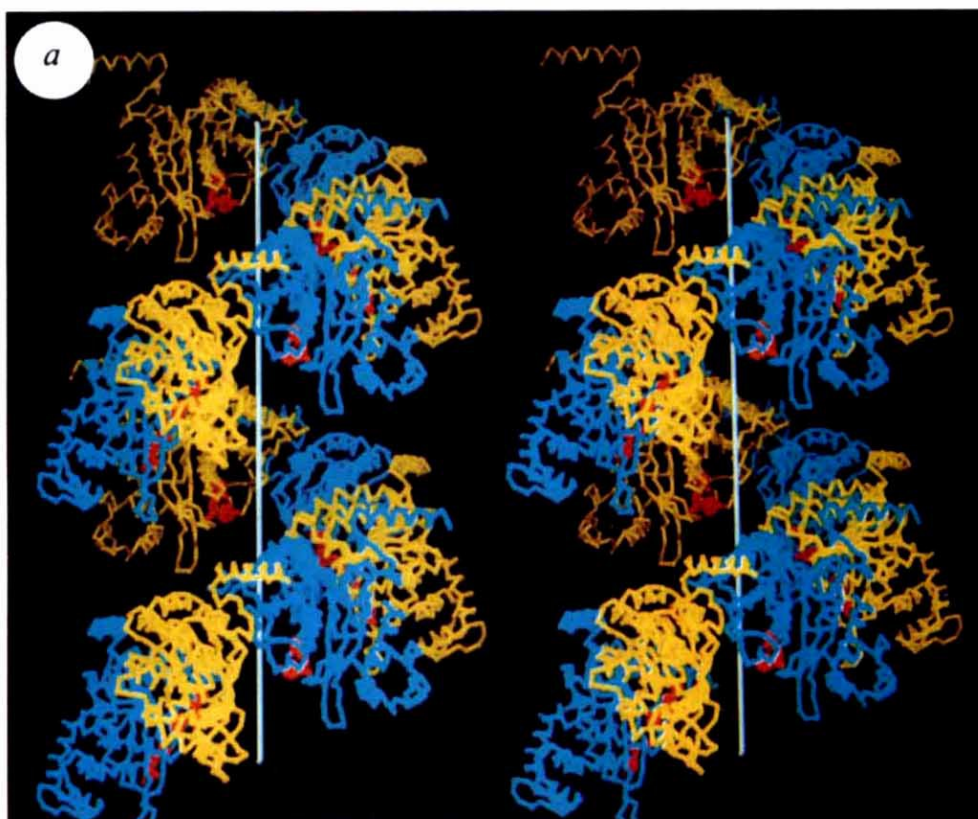


FIG. 3 The helical *recA* polymer observed in the crystal. *a*, Stereo α -carbon representation viewed parallel to the crystallographic 6_1 screw axis (shown in white). 12 monomers (two turns) are shown in alternating blue and yellow, with the bound ADP in red. The orientation of the upper left monomer is similar to that in Fig. 2.



Micro-targeting of microprojectiles to target areas in the micrometre range

Ingo Potrykus

A novel variation of the ballistic approach allows for reproducible targeting of micro-particle populations to areas of tissue as small as 100 μm .

MAJOR crop plants such as rice (*Oryza sativa*)¹, maize (*Zea mays*)², potato (*Solanum tuberosum*)³, tomato (*Lycopersicon esculentum*)⁴, soybean (*Glycin max*)⁵, oilseed rape (*Brassica napus*)⁶, and forage grasses (*Festuca arundinacea*)⁷ have been transformed, and others such as wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) probably will follow soon. Of the various approaches to gene transfer, four have led to the production of transgenic plants⁸. These are (1) *Agrobacterium*-mediated gene transfer, (2) protoplast-based direct gene transfer, (3) biolistics — gene transfer on the basis of accelerated micro-particles, and (4) micro-injection.

Each of these techniques has its specific merits and drawbacks, and there is no technique that would be applicable for any given gene transfer problem. For studies in basic research, gene transfer to plants is probably no longer a serious technical problem. The situation is, however, considerably different with regard to the application of gene transfer technology to practical problems in agricultural plant biotechnology.

Problems of practical application

For the efficient application of gene technology in plant breeding, it will be important to have methods available that work routinely and efficiently with most varieties of most crop plant species. This is not the case with the methods listed above. *Agrobacterium*-mediated gene transfer is not as yet (and probably will not be) possible with cereals and interesting varieties of herbaceous dicots. Protoplast-dependent direct gene transfer is possible with cereals, but only with varieties in which plant regeneration from protoplasts is possible. Biolistics, a straight-forward physical method, has few limitations but is inefficient in cases where plants do not provide large populations of, or easy access to, totipotent cells. Micro-injection offers the advantage of enabling the targeting of totipotent cells, where they are the limiting factor, but is technically demanding and still far from being used routinely.

The great strength and, at the same time, the reason for the limitations of the gene transfer methods discussed, is their

efficient use in *in vitro* culture systems, which are the basis for plant regeneration from somatic cells. The plant varieties that are most difficult to transform are those that contain few or no cells that can be regenerated to plants *in vitro*.

A way out of this dilemma could come from the transformation of 'germline'-type cells *in situ*. Considerable time has been invested in approaches of this kind but, with a few exceptions, there has been little success. It was one of the great advances from biolistic approaches to demonstrate that shoot tips of seedlings can be used for the production of transgenic offspring⁵.

Shoot meristem as a target

Shoot meristem, the tiny group of cells from which the entire plant develops, is a structure of outstanding interest for those researchers aiming to develop gene transfer methods that are applicable to any given plant. The 50–100 tiny cells of the shoot meristem grow to fertile plants in every given plant species. Integrative transformation in these few cells should guarantee the recovery of transgenic offspring. Transformation of these cells should be possible with *Agrobacterium*, micro-injection and ballistic techniques. *Agrobacterium*-based approaches as yet have not been successful (with the exception of *Arabidopsis* seed incubation⁹ where transformation, however, probably does not occur in the shoot meristem). The low efficiency of micro-injection^{8,10} may be due to technical difficulties and low competence for integrative transformation of meristematic cells. The relative low efficiency of biolistics is surprisingly high considering the fact that shoot meristems are tiny (about 100 μm) and the biolistic particles hit large target areas at random. Approaches using traditional biolistic designs solved the problem of hitting a tiny structure in a large target area with a random population of particles by placing large numbers of targets into the target area⁵.

Micro-targeting technique

An alternative design, which combines advantages of the micro-injection technique (the capacity to predict the position of delivery) with advantages of the biolistic

technique (the ability to achieve multiple hits with one event) has recently been developed by Sautter *et al.*¹¹. The device, which was developed specifically for biolistic micro-targeting of shoot meristems, fulfils all the criteria that are necessary for the predictable and routine acceleration of particle populations into the cells of any given shoot meristem under conditions that will not interfere with subsequent development of the meristem. During the course of development of the 'micro-targeter', it was necessary to overcome the following technical problems: (1) particles must be accelerated to target areas of variable size ranging between 100 and 200 μm , (2) the particles of any given population must be of identical size, (3) they must arrive at the target as individual particles, (4) their penetration into the target must be predictable and variable, and (5) the particles have to carry DNA which is freely accessible within the cell. The latter was achieved by developing a method for the preparation of gold particles from solutions of gold salts by the addition of photographic developer (parameters such as ratio of mixing and time of development determine the size of the particles). Clumping of particles is prevented by avoiding any kind of 'macro-projectile'. Instead, an aerosol is created from suspensions of gold particles in DNA-containing water. The aerosol is then accelerated in a Pitot's tube (see Fig. 1a) by the same gas-pressure pulse that created the aerosol. Acceleration occurs in a capillary (or 'restriction'), the length of which determines the impulse, the width the target area (see Fig. 1b). Accurate aiming is achieved with the help of a line-crossing in a microscope (see Fig. 2).

As each physical parameter of the system is variable within a wide range, the micro-targeting system can, therefore, easily be adjusted and optimized to suit the specific requirements of shoot meristems from different plant species. To prepare and deliver one 'shot' takes 10 seconds. The particles penetrate to deeper cell layers of the meristem (this is essential because sexual offspring will be produced from only the second cell layer). Plenty of particles can be targeted to one meristem, thus allowing for the delivery

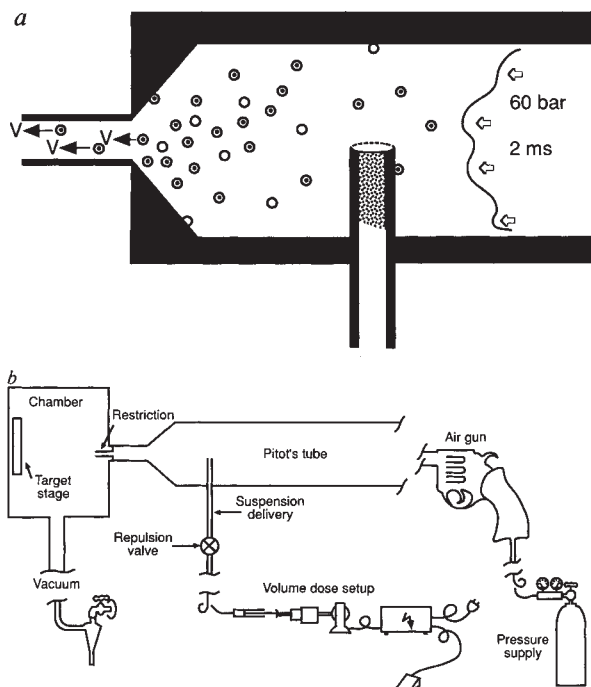


FIG. 1 Schematic views of the micro-targeter. a, In Pitot's tube, 20 nl of a gold/DNA suspension is dispersed by a pressure pulse. The particle-containing droplets are accelerated in a capillary (restriction). b, Total view of the set up. A simple airgun is used to produce the pressure pulse. The particle jet leaving the restriction is directed towards the tissue mounted on a movable target stage.

of DNA statistically to every cell of a meristem. Treated shoot meristems regenerate with high frequencies to fertile plants. Integrative transformation in an experimental cell-culture system containing cells competent for integrative transformation was very high¹¹. Marker genes have been expressed in shoot meristems of cereal seedlings. And, particles have been accelerated into shoot meristems of wheat (*Triticum aestivum*), Sorghum (*Sorghum bicolor*), and other graminaceous species, although data on integrative transformation are not yet available (C. Sautter *et al.*, personal communication).

Future prospects

As micro-targeting is not limited to shoot meristems and DNA, it may, therefore, find application in areas other than applied gene technology, such as basic

studies in plant biology. However, recovery of transgenic chimaeric plants and transgenic offspring therefrom depends not only on the delivery of DNA into and the regeneration of plants from shoot meristems, but also on whether or not there is integrative transformation in the meristematic cells. Unfortunately, we have to account for the possibility that meristematic cells may dispose of molecular mechanisms which prevents integration of foreign DNA^{8,10}. With its capacity to accelerate routinely populations of DNA-carrying particles into shoot meristems, micro-targeting will either be successful and lead to routine transformation, or it will provide a system to study whether or not meristematic cells have such a preventive mechanism. If so, then it will allow us to search for ways to circumvent such a mechanism. □

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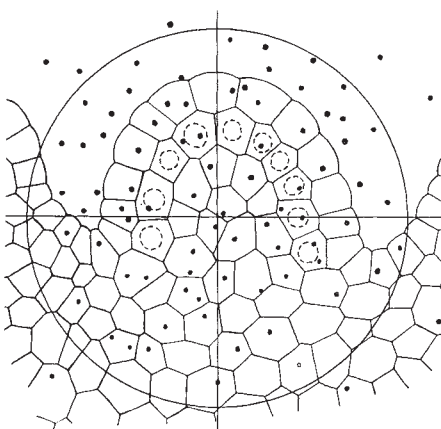


FIG. 2 Shoot meristem of a cereal seedling and particle distribution under the line-crossing, which is used for aiming purposes. The ring characterizes the targeting area of about 150 µm. The second cell layer (basis for sexual offspring) is indicated by drawn-in nuclei.

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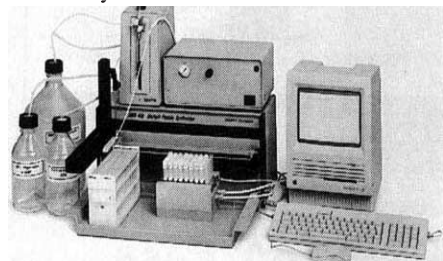
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Reader Service No.19

Product lines for the life sciences

Researchers will gather in Houston, Texas next week for the meeting of the American Society for Biochemistry and Molecular Biology. Featured products will include a coldroom-proof, UV/visible LC detector with a built-in recorder and peak sensor and a new dual-function thermostable enzyme.

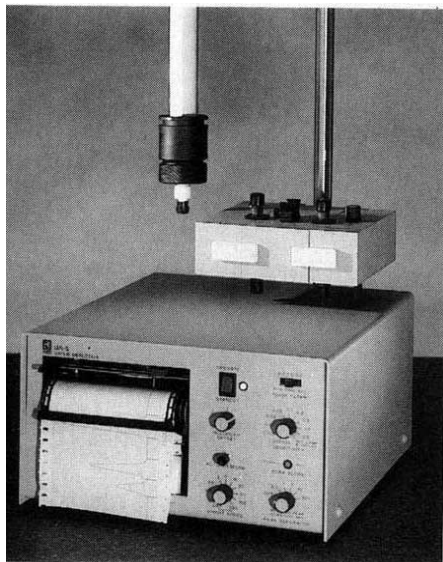
GILSON Medical Electronics has designed a **multiple peptide synthesizer**, the AMS 422, which is capable of simultaneously producing up to 48 different peptides, each consisting of up to 20 amino acids (*Reader Service No. 101*). The company claims that the low cost per peptide primarily is due to the speed of the instrument, the result of Fmoc chemistry and a flow-through design. Peptides are produced in reaction columns using *in situ* activation of amino acids. A set of 48 peptides can be synthesized in about 48 hours. The company says that low per-peptide cost also can be attributed to the efficiency of the AMS 422 during synthesis. The instrument optimizes amino acid coupling with a high reagent concentration; this speeds reactions and minimizes consumption of expensive reagents, Gilson says. Reactions also are enhanced



AMS 422 — 48 peptides in one go.

by the addition of a second solvent during coupling and other automatic functions that compensate for the special characteristics of some amino acids. By using a vacuum aspiration system to remove solvents and reagents, column washing time is kept to a minimum, and is only ten per cent of the total cycle time. Stop by booth 714.

Isco's UA-6 **UV/visible liquid chromatography detector** includes a detector, recorder and fraction-cutting peak sensor in a unit that is only 28-cm wide (*Reader Service No. 102*). Its remote, dual-beam optical unit is easily positioned near the LC column outlet to optimize plumbing. Twelve interchangeable flow cells provide optimum performance for low pressure LC (from research-scale to high flow), HPLC and microbore. In addition to the standard 254-nm and 280-nm wavelengths, fourteen other wavelengths span the spectrum from 214 nm to 660 nm. Other design features include anti-condensation protection for trouble-free operation in humid conditions, a peak sensor that controls practically any fraction collector so that peaks are cut at true



Coldroom-proof UV/visible LC detector.

beginnings and ends, and a built-in, 10-cm recorder that provides event marking along with accurate chromatographic output at a wide range of chart speeds. Isco will be in booth 1034.

Axon Instruments has developed **CyberAmp**, a computer-controlled **signal conditioner, anti-alias filter and sensor platform** (*Reader Service No. 103*). Each CyberAmp channel has differential inputs, a bandwidth from d.c. to 50 kHz, variable low-pass filtering by a high-order Bessel filter, variable gain up to 20,000, a.c./d.c. coupling, notch filtering and automatic baseline correction. A variety of plug-in SmartProbes, containing computer-readable calibration information, function as voltage amplifiers, picoammeters and adapters to many commercially-available transducers. Controlled via a serial port, the CyberAmp comes with DOS control software and a programmer's library. The CyberAmp is supported in the data acquisition software of Axon Instruments and other companies. This device, Axon says, is an 'intelligent' front end to engineering, scientific and biomedical data acquisitions systems. The eight-channel (380) and two-channel (320) CyberAmp systems are \$5,200 and \$2,400 (US), respectively. Axon Instruments will be in booth 942.

If your research involves the measurement of fluorescence, phosphorescence, bio- or chemiluminescence, then Perkin-Elmer's new Model LS-50B **luminescence**

spectrometer may be of interest, particularly to analysts in the fields of biochemical and environmental analysis (*Reader Service No. 104*). Based on the LS-50, the upgraded model features several design enhancements. Improved resolution, a remote start function and a fast data-collection rate permit the study of fast reactions using stopped-flow techniques, Perkin-Elmer says. In addition, kinetic data from four thermostatted cuvettes can be collected automatically using a wavelength program. Intracellular biology applications software allows the operator to study changes in concentration of intracellular calcium and pH using FURA-2 and BCECF. A new coverslip accessory simplifies the study of cells immobilized on coverslips and can be used with the thermostatically-controlled stirred-cell holder. A wide range of computer-controlled accessories allows the measurement of many differing samples, including liquids, solids such as powders and films and suspensions of living cells. See booth 326.

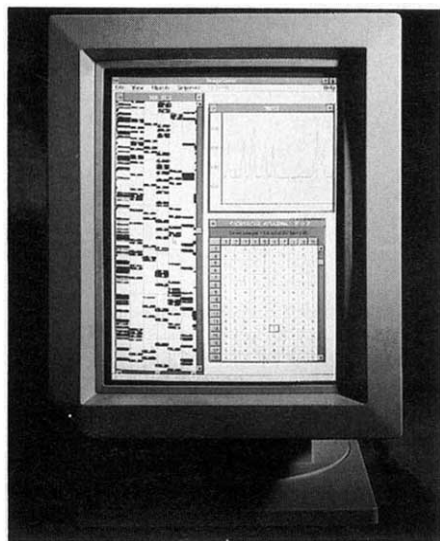
Molecular marketplace

Two functions, one enzyme — stop by booth 1053 and find out more. TET-z is the new **dual-function, thermostable enzyme** from Amersham that has both DNA polymerase and reverse transcriptase activity (*Reader Service No. 105*). Isolated from a strain of *Thermus thermophilus*, TET-z can be used for RNA amplification, performing both the synthesis of first-strand DNA from RNA and subsequent amplification. Amersham points out that the thermostable reverse transcriptase activity is useful when the RNA template has secondary or tertiary structure, as elevated temperatures may be used to produce full-length cDNA. TET-z also can be used to sequence ssDNA, nDNA and RNA. According to Amersham, studies have shown that TET-z is very stable in amplification procedures: routinely 40 cycles of amplification can be carried out with no observable loss in the efficiency of the enzyme. Available in either 50 or 300 units, TET-z is supplied with both reverse transcriptase buffer and DNA polymerase.

The new Colony Images Kit from United States Biochemical is designed to **screen DNA clones made in both plasmid and phage libraries using chemiluminescent detection** (*Reader Service No.*

106). Colonies or plaques are lifted from culture plates with Colony Images nylon membrane and subsequently treated with 10 per cent SDS to denature cellular proteins. Target DNA is denatured with sodium hydroxide, neutralized and then fixed to the membrane. Biotinylated probes made with Random Primed Images are hybridized to specific target sequences within the vectors and chemiluminescent detection is performed using streptavidin-alkaline phosphatase and Lumi-Phos. The membrane is exposed to X-ray film and positive colonies can be identified. USB says that the kit delivers low background with respect to negative colonies or plaques. The \$250 (US) kit includes colony lift disc-shaped membrane, 5× blocking buffer, streptavidin-alkaline phosphatase conjugate and Lumi-Phos 530. See booth 1330.

Drop by booth 1814 and see the new full-page pivot and SVGA monitor for use with the PhosphorImager and Computing Densitometer from Molecular Dynamics (*Reader Service No. 107*). The **advanced graphics system** displays images in 224 levels of grey for realistic 'film-like' images, as well as 256 colours. The 1,024 × 768 resolution provides 65 per cent more screen information than a standard VGA monitor, the company says. ImageQuant, the company's Windows 3.0-compatible quantitation software is fully compatible with the advanced graphics system, taking full ad-



Full-page pivot and SVGA monitor. vantage of its display capabilities. By rotating the display, images and spreadsheets as wide as 28 cm can be accommodated; the portrait position can be used for DNA sequencing gel images and full-page text displays.

A variety of factors can dramatically affect the fidelity of initiation and translation efficiency. Salt concentration, in particular, can be difficult to control in

many lysates. In order to **optimize the production of proteins from mRNA**, Promega has introduced the Salt Select Rabbit Reticulocyte Lysate System (*Reader Service No. 108*). With Promega's system, the concentration of Mg^{2+} , K^{+} and DTT can be fine-tuned for a specific message. A rapid, nonradioactive luciferase assay is included as a control. Promega says that sufficient reagents and a detailed protocol are included for optimizing thirty 50- μ l translation reactions. See Promega's latest products in booth 1435.

CLONTECH now offers **glyceraldehyde 3-phosphate dehydrogenase (G3PDH) and transferrin receptor PCR amplimers and cDNA probes** — alternatives to β -actin as a control for a variety of PCR and other laboratory procedures (*Reader Service No. 109*). In RT-PCR and Multiplex PCR experiments, the company's G3PDH Amplimer Set and Transferrin Receptor Amplimer Set are used along with gene-specific primers as a control to verify gene expression and regulation, to confirm uniform first-strand cDNA synthesis efficiencies and to semi-quantitate gene expression levels. In many cases, G3PDH is preferred over β -actin because, unlike β -actin, G3PDH transcription levels generally do not change in response to inducing agents such as cytokines and tumour-promoting phorbol esters, and levels remain fairly consistent among most tissues. Transferrin receptor is useful as a control in RT-PCR as, unlike β -actin, its low level of expression will not overwhelm the expression of many amplified target genes. G3PDH and Transferrin Receptor cDNA Probes can be used along with Control Amplimers to confirm PCR results, or alone to verify equal loading of RNA in northern and RNA dot blots, and as a control in Multiplex northern hybridizations and nuclear transcription run-off assays. The G3PDH and Amplimer Sets allow convenient and specific amplification of cDNA, CLONTECH says. Each Set consists of 5' and 3' primers sufficient for 100 specific amplifications, positive control DNA and a detailed protocol. The G3PDH and Transferrin Receptor Probes are purified cDNA inserts that are ready for immediate labelling. They are provided as 1- μ g quantities, sufficient for at least 20 standard hybridizations. See booth 1520.

Power packs

Fisher Scientific has introduced the new FisherBiotech **power supply**, which combines ease of operation with the versatility required for complex electrophoretic techniques (*Reader Service No. 110*). The power supply incorporates several advanced programming func-



FisherBiotech power supplies.

tions, accessed through user-friendly menus, for techniques such as isoelectric focusing and for multi-stage PAGE and SDS-PAGE experiments, where sample introduction is at lower voltage levels followed by separations at much higher field strengths. Three models are available: 1,000 V, 2,000 V and 3,000 V. Each has four output jacks to accommodate multiple chambers. Users can control experiments by volt-hour integration, milliamp-hour integration, watt-hour integration and cell-resistance monitoring. Up to 150 parameters can be stored in the memory. Pre-set and actual conditions appear on the four-line LCD. The power supplies will also run multi-step programmes, linking up to ten routines. Ten such multi-step sequences can be stored in the memory. Fisher will be in booth 1614.

With three models to choose from, the new Series 700 **power supplies** from E-C Apparatus are designed to be simple to use for routine runs yet capable of handling demanding multi-step procedures (*Reader Service No. 111*). The Series 700 includes 1,000-V, 2,000-V and 3,000-V versions — all of which feature constant



Series 700 power pack.

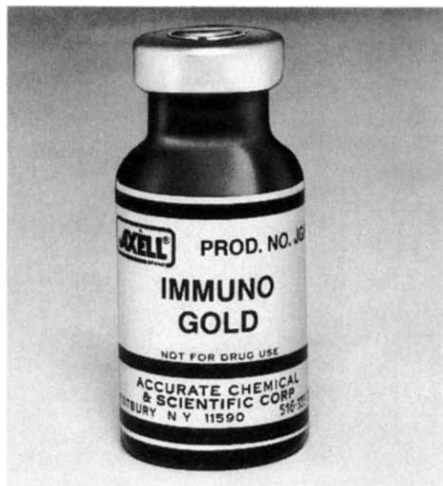
voltage, constant current and constant power operating modes with automatic crossover. Each power supply has a selection of programming options, including timers, volt-hour integrators, resistance monitors, single- and multi-step programme storage. Visit E-C Apparatus in booth 1214.

Ideas for the immunologist

Advanced Magnetics has introduced an interleukin-1 β microtitre 'sandwich' enzyme immunoassay for the **quantitative determination of IL-1 β levels in human serum or cell culture supernatant** in less than four hours (*Reader Service No. 112*).

IL-1 β is a polypeptide cytokine that mediates ranges of biological activity, including the synthesis of the acute-phase proteins by hepatocytes, chemotaxis of polymorphs and release of PMNs from blood and bone marrow. A precision-coated well plate is supplied with each kit to provide both consistency and flexibility, and prediluted, lyophilized standards eliminate dilution variability, Advanced Magnetics says. The sensitivity of the assay in buffer is 1.6 pg ml⁻¹; 4.3 pg ml⁻¹ in serum. The IL-1 β test kit, which is specific for bioactive human IL-1 β , contains sufficient reagents for 96 tests, including standard curves. See booth 1026.

A full range of **colloidal gold reagents complexed to affinity-purified antibodies and other proteins** can now be obtained from Accurate Chemical and Scientific (*Reader Service No. 113*). Immunogold reagents permit labelling with a full range of primary antibodies, without background from crossreacting antibodies. In addition, their gold-recombinant Protein A/G binds to more species than either Protein A or Protein G alone. Two grades are available: an LM and EM grade. The LM grade provides a 4-nm particle and is designed for use when penetration during pre-embedding procedures is a concern. The LM grade is available freeze-dried and can be re-frozen after being reconstituted in water. The EM grade uses a larger particle in



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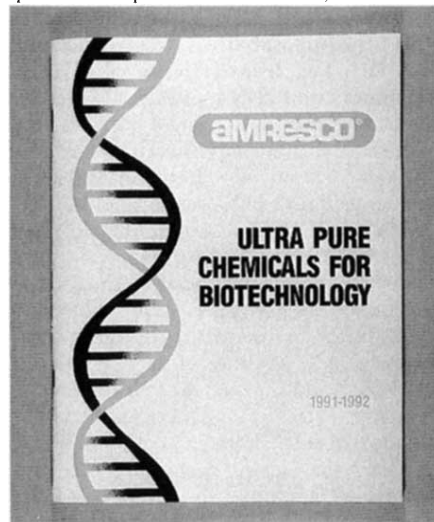
combination with a newly developed stabilizing solution, which helps to maintain a high degree of activity, Accurate says. Accurate also offers an Immunogold Silver Enhancement Reagents kit. See booth 1027.

Off the shelf

The new **biotechnology product handbook** for 1992 is now available from Polysciences (*Reader Service No. 114*). The handbook features an alphabetical product index, a biotechnology product

highlight section and a 32-page technical applications guide. Areas covered include electrophoresis, chromatography, ultrafiltration, blood-cell isolations, silver staining and dialysis. In addition, the guide features Polybead polystyrene microparticles, fluorescent magnetic and functionalized microspheres for diagnostic applications. See booth 1516.

AMRESCO announces the availability of its 72-page 1991-1992 **catalogue of ultrapure chemicals for biotechnology** (*Reader Service No. 115*). The new line up includes pre-mixed buffers, saturated

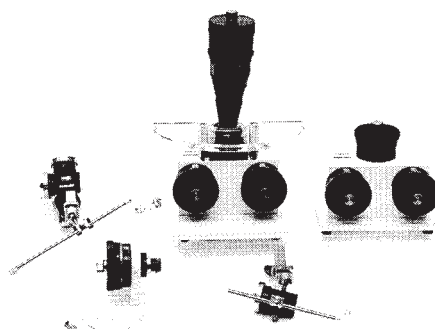


Biotech buyer's guide.

phenols and acrylamide stock solutions, agarose gels, dyes and stains. An abbreviated Certificate of Analysis for each product is designed to facilitate selection. See booth 127.

Micromanipulation

For maximum stability during the long recording times involved in patch-clamp studies, Narishige recommends its MW Series of **micromanipulators** (*Reader Service No. 116*). Drift is controlled using a water-filled, rather than an oil-filled, hydraulic system. The positioning controls are graduated to a 0.2- μ m movement of the micropipette, Narishige says. Two three-axis versions are available in the MW Series: the MW-3 unit for conventional three-axis positioning and



Water hydraulic micromanipulators.

the MW-2, which has, in addition, a joystick control that can be used to control all three axes. Completing the line up, is the MW-4 single-axis manipulator. All three can be connected to most makes of microscopes using the Narishige range of mounting adaptors and coarse mechanical positioners. See booth 1127.

Adams and List says that by using its **2PK pipette perfusion kit** it is possible to perfuse internally patch pipettes during recordings without introducing excess electrical noise (*Reader Service No. 117*). All the equipment necessary to perform the technique is included in the kit. The technique involves heating and pulling a quartz perfusion pipette and inserting it into the patch pipette. The unpulled end of the quartz pipette is attached to thin tubing, which is used to transfer the drug to the tip of the pipette. A precision vacuum generator is provided to control the amount of substance to be perfused. Catch up with Adams and List in booth 133. □

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